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The Effects of Theophylline and 8-Cyclopentyltheophylline on the Respiratory Response to Carbon Dioxide in Neonatal Rats

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ABSTRACT

Premature infants are often plagued with respiration problems ranging from periodic breathing to apnea. These respiratory complications can be a result of underdeveloped respiratory organs, immaturity of the brainstem respiratory control center, genetic irregularities, or a combination of all three. This study sought to increase respiration with the administration of methylxanthines, which are respiratory stimulants, coupled with carbon dioxide (CO_2), also a respiratory stimulant. Neonatal rats aged 4 to 7 days old were used to mimic premature infants' response to the interaction of methylxanthines and CO_2. Before beginning the respiratory studies, sections of a 4- and 7-day-old rat brainstem were examined to compare the differences in development. The 4-day-old rat brain had large folds (folia), while the 7-day-old rat brain lost those folds and was much denser in neuroglial and nerve cells. These differences show that as the rat matures, the brain also matures because the folds disappear, meaning that the brain is growing and new cells are synthesized as a part of this growth. After establishing this difference in brain development between the youngest and oldest rats, various doses of two different methylxanthines, theophylline (THEO) and 8-cyclopentyltheophylline (CPT), were injected into neonatal rats and paired with CO_2 percentages ranging from 1 to 6%. The interaction of the drug and CO_2 was observed over a 45-minute period. Each rat was placed into a body plethysmograph that was connected by plastic tubing to a pneumotachograph to measure the rat's respiration. A control period of normal breathing for 5 minutes followed in order to establish a baseline respiration, which was followed by a CO_2 –response test involving exposure to increasing percentages of CO_2 from 1 to 6%, delivered at 2-minute intervals. This same procedure was repeated in 15-minute intervals

until the 45-minute period concluded. CO_2 exposure produced a consistent increase in minute ventilation, tidal volume, and mean inspiratory flow, but not in respiratory rate, all of which were independent of the drug response. Although there was not an overall significant difference between doses of THEO, the highest dose, 40mg/kg, showed significant increases in minute ventilation, tidal volume, and mean inspiratory flow when paired with 5-6% CO_2. The dose of 10 mg/kg of THEO also showed increases in minute ventilation and tidal volume at higher CO_2 percentages. In contrast, CPT showed no significant increases in respiration at any dose. Interestingly, THEO doses were significant though CPT is the more potent form of the drug and has a higher affinity for adenosine A_1 receptors. In summary, CO_2 alone produced increases in minute ventilation, tidal volume, and mean inspiratory flow, and when paired with a dose of 40 mg/kg of THEO, these parameters increased further, showing that the highest doses of THEO paired with the highest doses of CO_2 produce a significant increase in respiration.

INTRODUCTION

Preterm infants often suffer difficulties in respiration when they are first born due to immature brain and respiratory organ growth. The respiratory system begins forming from the ectoderm and mesoderm, with the organs developing during the fourth week of gestation (Crawford, 2011). The organs of the respiratory tract can be divided into two sections, conducting and respiratory (Crawford, 2011). The conducting section includes the nasal cavity, pharynx, larynx, trachea, bronchi, bronchioles, while the respiratory section includes the bronchioles, alveolar ducts, alveolar sacs, and alveoli (Crawford, 2011). Premature infants can suffer many respiratory problems as a result of these organs not being developed fully while the baby is still in the womb (Crawford, 2011). Starting with the infant's relatively large head in comparison to its short neck, this relationship results in flexion, compromising the flow of air in the trachea (Brouillette *et al.*, 1990; Crawford, 2011). In addition, nostril size can be small and become even smaller if blocked by mucus. Tongue size, being relatively large compared to the size of the infant mouth, also decreases breathing (Brouillette *et al.*, 1990; Crawford, 2011). Both the larynx and the trachea are short and small, which means that a small obstruction can impair breathing (Brouillette *et al.*, 1990; Crawford, 2011). The development of the ribs plays a significant role in breathing as well. Rib position is horizontal, and the thorax shape is circular (which changes to an ellipse later in development), and the chest wall is thin with little muscle to stabilize it (Crawford, 2011). In addition, the diaphragm is less efficient in contracting because of its flat shape (Crawford, 2011). Consequently, the cartilaginous rib cage becomes deformed easily, putting more stress on the diaphragm and chest wall muscles, which usually results in the chest wall collapsing and the

diaphragm being less sufficient in inspiration and expiration (Brouillette *et al.*, 1990). The chest deformity occurs during REM sleep since the muscle activity is being inhibited (Brouillette *et al.*, 1990).

In addition to the respiratory system, the brain plays a vital role in respiration. The medulla oblongata, a part of the brainstem located at the base of the brain where it thins into the spinal cord, is the regulation site for breathing, heart rate, and blood pressure, as well as minor functions such as mediating sensory impulses, endocrine secretions, and general awareness (Chiasson, 1988). The brainstem is arguably the simplest, but most important part of the vertebrate brain since in some animals (i.e., lizards) it is the largest portion of their brain. Action potentials originating from neurons of the brainstem travel along the vagal and phrenic nerves (Leiter & St. John, 2004). These nerves show increased levels of activity when breathing is not normal, which shows evidence of the brain's connection to respiration (Leiter & St. John, 2004). In particular, the phrenic nerve carries signals from the brainstem to the diaphragm, and the vagus nerve influences both respiratory rate and lung tidal volume by innervating the stretch receptors of the lungs (Akay, 2005). The information gathered from these nerves then travels back to the brainstem where a decision is made on how to optimize breathing (Akay, 2005). If the brainstem and nerves are underdeveloped, this process won't be as efficient and the infant will struggle to breathe for a longer period of time. For instance, kittens born full term have 10% of vagal nerve fibers at birth and 50% about 60 days postnatal, and the diameter of these fibers continues to increase at a rate of 0.2 μm/day after birth (Akay, 2005). If these kittens were born preterm, they would be less developed and most likely have more difficulty with respiration.

When infants are born preterm, the respiratory organs and brain are underdeveloped causing breathing problems. One minor difficulty in respiration is known as periodic breathing, which is described as 3 or more pauses of more than 3 seconds with less than 20 seconds between pauses (Oliveira *et. al.*, 2004; Brouillette *et al.*, 1990). Normal pauses in infants breathing range from 6-10 seconds (Brouillette *et al.*, 1990). An even more serious condition is apnea, defined as pauses in breathing lasting 20 seconds or longer (Brouillette *et al.*, 1990). When paired with hypoxia, a deficiency in the amount of oxygen in the bloodstream, and bradycardia, a slow heart rate, apnea can be quite severe (Bleul *et. al.*, 2010; Schmidt *et. al.*, 2006). Primary apnea occurs when hypoxia is not treated by the stimulation of respiration (Bleul *et. al.*, 2010). Secondary apnea occurs if the condition worsens by neglecting treatment further, which leads to failure of the respiratory center in the brain and no oxygen supply to the brain, leading to risk of death (Bleul *et. al.*, 2010). There are three types of apnea including central apnea, where there is a cessation of respiratory effort for more than ten seconds; obstructive apnea, characterized by continued inspiratory efforts against an occluded airway; and mixed apnea, which is a combination of the first two types (Carley & Radulovacki, 2008). In any case, if these diseases are not treated, serious complications or even death may result.

One type of preliminary treatment is the administration of carbon dioxide (CO_2) to those suffering from apnea. Carbon dioxide naturally causes infants to increase their minute ventilation and tidal volume, due to its respiratory stimulating effect, but CO_2 alone can only do so much when the infant is preterm. One group of investigators hypothesized that a decreased sensitivity to CO_2 correlates with younger age, which

makes sense since the younger the infant is, the less developed the breathing organs and brain function are (Rigatto *et. al.*, 1975). This hypothesis was supported by the CO_2 response curve slope, which increased by 42% from 32 to 37 weeks gestation and 62% from 2 to 27 days of age (Rigatto *et. al,* 1975). These findings support the idea that as the infant ages, its respiratory center in the brain as well as its respiratory organs mature and become more sensitized to CO_2. The increased sensitivity to CO_2 results in a greater response to CO_2, which also facilitates more consistent and deeper breathing (Rigatto *et. al.,* 1975). This study stimulates questions about whether this sensitivity to CO_2 seen in infants can be manipulated and enhanced if paired with other treatment options.

One option that could enhance CO_2 effects would be coupling its use with methylxanthine drugs that act as respiratory stimulants by antagonizing adenosine A_1 and A_2 receptors. Adenosine acts as a neurotransmitter with numerous biological actions, including respiratory depression (Mokry & Mokra, 2013; Shamin, 1989). An overactivity of central adenosine mechanisms is linked to irregular breathing or apnea in premature infants (Mokry & Mokra, 2013). By administering methylxanthines, the A_1 and A_2 receptors are bound to the drug and thus cannot continue on their normal path of bronchoconstriction (Mokry & Mokra, 2013; Brunton *et.al.,* 2006; Shamin, 1989). The xanthine drugs widen the bronchi, relax airway smooth muscle, and enhance breathing (Brunton *et.al.,* 2006). Theophylline (THEO) is a methylxanthine that is often used to treat preterm infants with ventilatory problems. However, this drug can be even more effective in treatment by the addition of different substituents to the 1, 3, and 8 positions of the xanthine nucleus, which would increase the potency (Shamin, 1989). However, additions greater than propyl at the 1 and 3 positions reduce activity at both adenosine A_1

and A_2 receptors (Shamin, 1989). By adding cycloalkyl groups to the 8 position of THEO, the potency increases, while it increases the activity at adenosine A_1 receptors with only a small increase in activity at adenosine A_2 receptors (Shamin, 1989). Thus 8-cycloalkyl moieties are potent and selective for adenosine A_1 receptors. The difference between the drug's ability to inhibit A_1 and A_2 receptors could be because there are many subclasses in each receptor and this range of subclasses has many different recognition sites for the drug to bind to (Shamin, 1989). In contrast to 8-cyclopentyltheophylline (CPT), administration of THEO requires a higher dose, which often puts the infant at higher risk for negative side effects such as increased heart rate, irritability, seizures, and vomiting. This increased activity of the heart and central nervous system will cause the infant to burn more calories and use more energy, while the gastrointestinal distress will interfere with nutrition (Brunton *et. al.*, 2006; Aranda *et. al.*, 1986; Brouillette *et. al.*, 1990). THEO is bound to plasma proteins to a greater extent than is caffeine (another common xanthine derivative), and the fraction bound declines as the concentration of methylxanthine increases, which explains why a larger dose of theophylline is needed (Brunton *et. al.*, 2006). Caffeine is another drug option for the treatment of neonatal apnea and has similar effects to THEO and CPT (Brunton *et. al.*, 2006).

Aranda *et. al.* (1986) found that when methylxanthines were used in conjunction with additional CO_2, there were increases in ventilation, tidal volume, and ventilatory response to CO_2. In addition, Uauy *et. al.* (1975) found that THEO increased minute volume and sensitivity of the central response to hypercapnia (elevated CO_2). Both of these studies provide concrete evidence that has led to my hypotheses. In order to mimic the interaction of THEO and CPT with the ventilatory response to CO_2 seen in preterm

infants, newborn rats were chosen because they serve as a good model for breathing control of premature infants with a diminished respiratory response to CO_2. Rigatto *et. al.* (1981) explains that rats and humans share similar basic physiology, similar organs, similar body plans, and control body chemistry by secreting hormones, which make the infant rat a satisfactory substitute for premature infants. With that in mind, my hypotheses include: (1) the administration of methylxanthines such as THEO and CPT will increase the newborn rat's ability to respond to CO_2, which in turn will increases respiration; (2) CPT will show a more significant effect on respiration than THEO since it is the more potent methylxanthine at adenosine A_1 receptors; (3) CO_2 alone will also increase respiration, but not as significantly as when paired with a methylxanthine. These comparisons will help to identify benefits of both THEO and CPT, as well as effective and safe doses of each drug. My overall research goals are to further the understanding of the effects of methylxanthines on respiration and to identify a safe, effective respiratory stimulant drug that can help treat infant apnea.

METHODS

Animals

The subjects used for this experiment were newborn rat pups obtained from a breeding colony of Sprague-Dawley laboratory rats at Eastern Illinois University in Charleston, Illinois. The colony was kept in a temperature controlled building within a separate air filtering system. The newborn rats were selected randomly within the desired ages ranging from 4 to 7 days old because this age mimics the level of respiratory control of a preterm infant (Albers & McGilliard, 2012). When the rat was chosen the body weight, sex, age, and litter number were recorded.

Administration of Drugs and Carbon Dioxide

Respiration studies were performed on 4- to 7-day-old rats with three different variables available including two drugs (CPT and THEO) and CO_2. The studies were separated according to drug, with the CPT studies done first and the THEO studies done after. Both studies included three doses of a methylxanthine and increasing percentages of CO_2 paired with each dose. Each rat served as its own control, but there were also two saline control groups that received 0.9% sodium chloride instead of the drug and were exposed to CO_2 in the same manner as the drug-treated rats. Each treatment group consisted of 8 rats with a total of 64 animals (16 control (saline) rats, 24 THEO-injected rats, and 24 CPT-injected rats). Carbon dioxide levels ranged from 1% to 6% with the percentage increasing by one every 2 minutes. CPT doses started at a low dose of 320 μg/kg and continued to 640 μg/kg, and 1280 μg/kg. THEO doses were administered at 10 mg/kg, 20 mg/kg, and 40 mg/kg. These doses were chosen because previous studies in this lab established the dose range of THEO and CPT that produced respiratory

stimulation in newborn rats (Albers & McGilliard, 2012). The methylxanthines are poorly soluble in water and CPT has especially low water solubility (Shamin, 1989). Therefore, each drug was dissolved in a small amount of 1.0M NaOH and then diluted to the final concentration with 0.9% NaCl (with the pH being adjusted to about 8 in the process). The concentration was such that the drug could be administered in a volume of 5 µl/g body weight (*e.g.* 10 mg/kg rats were injected with a THEO concentration of 2 mg/ml). Because CPT was at the limit of its solubility, the highest dose (1280 µg/kg) was given as 10 µl/g of 128 µg/ml.

Animal Preparation and Equipment Used

After the doses of each drug were made, the animal was prepared. A rat was randomly chosen from the litter, where it was sexed and weighed. The animal was placed in a plethysmograph, which is a cylindrical container 8 cm long and 2.6 cm in diameter that confines the rat with a rubber seal around its neck and directs flow of displaced air through an outlet for measurement. The plethysmograph was placed in an environment stabilizing apparatus that consisted of a 6.5 cm internal diameter water-jacked cylinder that was heated to 35°C to maintain the animal's body temperature and sealed with rubber stoppers on both ends to control CO_2 concentration to which the rat was exposed (Fig. 1). The plethysmograph was connected to a Fleisch #0000 flow transducer (Phipps+Bird, Richmond, VA) by plastic tubing. This device, paired with a differential pressure transducer and carrier demodulator (Validyne Engineering, Northridge, CA) served as a pneumatotachograph, measuring the flow of air moving into and out of the cylinder as the rat breathed. Another plastic tube connected the environment stabilizing apparatus to the gas mixing apparatus, which consisted of a tank

of 8% CO_2, an air compressor (GAST, Benton Harbor, MI), a Model 252 airway gas monitor (Puritan-Bennett, Wilmington, MA), and a gas mixer (Matheson Gas Products, Basking Ridge, NJ). The gas mixer was operated by hand to control the percentage of CO_2. These values were measured on the airway gas monitor in percentages, with the standard oxygen percentage being between 18-21% and the CO2 percentage ranging from 0-6%. Before studies began, the pneumotachograph was calibrated by plunging air from a 20 ml calibration syringe through the flow transducer to see if the correct amount of air was being measured.

Respiratory Studies

After equipment calibration and drug doses were prepared, each rat was put through a preliminary control phase (without any drug) consisting of a baseline respiration, which was tested for 5 minutes followed by 2-minute intervals where the CO_2 percentage was increased by 1% increments in a range of 1-6%. Then, each rat was injected subcutaneously through the scalp with a drug (either CPT, THEO, or saline) without removing the rat from the plethysmograph. After that, another 5-minute baseline was administered, followed by 3 rounds of 2-minute intervals where the CO_2 percentage was increased again in a range from 1-6%. An injection was used as opposed to a capsule because the infant rat's digestive system isn't developed enough to metabolize a capsule, and the drug would take longer to be absorbed and affect the rat's respiration. In addition, the injection was given subcutaneously on the scalp because it was the most accessible region, while the rat was in the apparatus, and this location provided low risk of injury to vital organs. Respiratory rate and volume were recorded continuously, which was roughly every 15 minutes (the study stops at 45 minutes). The data collected were

transferred to a PowerLab. The measurements taken from the PowerLab included the respiratory rate (f), the tidal volume (V_T), minute ventilation (V_E), time of inspiration (T_I), time of expiration (T_E), and mean inspiratory flow (MIF; V_T/T_I). Upon completion of each experiment, the rat was returned to its mother.

Data were expressed as both raw averages and percent change compared to the rat's mean pre-drug control measurement, and CO_2-response curves were drawn for each drug dose. Percent change is valuable because respiratory data in unanesthetized animals are highly variable, and this removes the predrug variability in respiration between animals. In addition, the graphs were compiled by separating THEO and CPT, as well as separating the time intervals (control period, 15 minutes, 30 minutes, and 45 minutes after treatment added).

Treatment effects and CO_2 response were compared by two-way analysis of variance with repeated measures. Separate statistical analyses were conducted on each drug and each respiratory variable (*e.g.* THEO V_E). Each analysis included the range of CO_2 concentrations tested (0 to 6%) and times (predrug to 45 min postdrug). Statistical significance was determined at the $\alpha = 0.05$ level by two-way analysis of variance (ANOVA) with repeated measures since each rat served as its own control (Steel and Torrie, 1980). Multiple comparisons (*e.g.* comparisons between doses) were made using the Holm-Sidak method (GraphPad Software, 2014). The analysis of variance was performed using SigmaPlot 11.2 (Systat Software).

Rat Brainstem Dissection Technique

Four and seven-day-old rats were euthanized by exposure to CO_2 prior to dissection. Skin on the rat's scalp was removed immediately afterward using scissors,

and then the skull was cut from the base using scissors. This exposed the cerebellum and brainstem fully. The brainstem was removed and fixed in formalin-acetic acid-alcohol (FAA) for two days.

Rat Brainstem Histology

After fixing in FAA, the brainstem was transferred to a scintillation vial where it was washed in tap water and dehydrated in four changes of 70% ethanol for 1 hr each. The specimen went through more dehydration steps, containing five solutions with varying amounts of t-butyl alcohol (Table 1). The specimen remained in solution I for 12 hrs and solutions II-IV for 6 hrs each. The specimen went through three changes of solution V (pure t-butyl alcohol), where two changes were for 6 hrs each and one change was for 12 hrs. A small amount of eosin Y was added to the first change of solution V to stain the parts of the specimen that were transparent. Lastly, the specimen was placed in a 50:50 mixture of t-butyl alcohol and paraffin oil for 1 hr. Specimen were the transferred to paraffin, cast, and mounted onto blocks for sectioning (Ruzin, 1999). Sections were made on a rotary microtome at 10 ρm thickness (Ruzin, 1999). Ribbons were mounted onto slides using Haupt's adhesive (Ruzin, 1999). To remove the paraffin, sections were put through two changes of limonene for 5 minutes each. Rehydration was done using a graded series of ethanol. Sections were stained in Heidenhem's ironalum hematoxylin and then was placed in a solution of DI water with two drops of ammonia for 5 minutes and rinsed in DI water for 5 minutes (Guyer, 1936). Eosin was used as a counterstain for 20 minutes (Guyer, 1936).

Stained sections were dehydrated in graded ethanol series and cleared in limonene. Permount was used to attach the coverslip. Slides were examined with a light

microscope to compare the morphological differences between the brainstems of 4- and 7-day-old rats.

RESULTS

Respiration Studies

The trend for THEO studies showed a significant increase in V_E when 10 mg/kg and 40mg/kg of THEO were used when compared to the time and percent CO_2 (Fig. 2), while all treatments for CPT showed similar results whether in the control period or anytime after the treatment was added (Fig. 3). Looking first at V_E, there was no significant treatment effect for THEO ($p=0.529$, two-way ANOVA with repeated measures). However, there was a highly significant effect of time and % CO_2 ($p<0.001$), and there was a highly significant interaction between treatment (dose), time, and % CO_2 ($p=0.004$) (Table 2).

When multiple comparisons were made between doses of THEO and saline at different times and % CO_2 by the Holm-Sidak method, 10mg/kg of THEO produced an increased V_E compared to saline at 30 and 45 minutes at 6% CO_2 ($p=0.038$ and 0.004, respectively), and 40mg/kg of theophylline also increased V_E at 45 minutes at 6% ($p=0.008$).

Similar to THEO, CPT had no significant treatment effect ($p=0.166$, two-way ANOVA with repeated measures). However, there was a highly significant effect of time and % CO_2 ($p<0.001$), while there was no significant interaction between treatment (dose) and time and % CO_2 ($p=0.703$) (Table 3). Although no treatments of CPT were significant, there were some instances of treatments causing increases in V_E. For instance, 320 μg/kg of CPT increased V_E at 15 minutes and 3 and 5% CO_2 ($p=0.05$ and 0.010, respectively), 1280 μg/kg of CPT increased V_E at 15 minutes at 6% ($p=0.047$), and 640 μg/kg of CPT increased V_E at 15 minutes at 6% ($p=0.025$) and 45 minutes at 6%

($p=0.028$). There was a significant increase in V_E as more CO_2 was added and the time increased ($p<0.001$). It should be noted that any significant difference between any parameter and increased time and CO_2 represents the normal CO_2 response curve.

No treatments of THEO or CPT produced significant changes in f. Looking at THEO, there was no significant treatment effect in f ($p=0.350$), time and % CO_2 ($p=0.092$) and treatment interaction with time and percent CO_2 ($p=0.178$) (Fig. 4, Table 4). Similarly, CPT results for f showed no significant treatment effect ($p=0.943$). However, there was a highly significant effect of time and % CO_2 ($p<0.001$), while there was no significant interaction between treatment (dose) and time and % CO_2 ($p=0.548$) (Fig. 5, Table 5).

Tidal volume (V_T) showed a significant treatment effect for THEO at 40mg/kg ($p=0.006$) and a highly significant effect of time and % CO_2 ($p<0.001$), while there was no significant interaction between treatment (dose) and time and % CO_2 ($p=0.263$) (Table 6, Fig. 6). When multiple comparisons were made between doses of THEO and saline at different times and % CO_2 by the Holm-Sidak method, 40 mg/kg of THEO showed increased V_T at 15 minutes at 5 and 6% ($p=0.003$ and $p<0.001$ respectively), 30 minutes at 6% ($p<0.001$), and at 45 minutes at 2,3,5,and 6% ($p=0.009$, 0.015, 0.002, 0.001). In addition, 20 mg/kg of THEO showed some increases in V_T at 45 minutes at 2% and 5% ($p=0.004$, 0.033), but the treatment didn't produce an overall significant difference from saline.

In contrast, CPT treatments had similar V_T's and showed no significant differences in treatment (Fig. 7). There was no significant treatment effect for CPT ($p=0.840$). However, there was a highly significant effect of time and % CO_2 ($p<0.001$),

while there was no significant interaction between treatment (dose) and time and % CO_2 ($p=0.374$) (Table 7).

Time of inspiration (T_I) had no significant variance between THEO treatments (Fig. 8) and CPT treatments (Fig. 9). Time of expiration (T_E) also did not show any significant variance (Fig. 10, Fig. 11). Starting with the THEO treatments, there was no significant treatment effect for T_I ($p=0.783$). However, there was a highly significant effect of time and % CO_2 ($p<0.001$), while there was no significant interaction between treatment (dose) and time and % CO_2 ($p=0.794$) (Table 8). In addition, there was no significant treatment effect for T_E ($p=0.567$). Similar to T_I, there was a highly significant effect of time and % CO_2 ($p<0.001$), but no significant interaction between treatment (dose) and time and % CO_2 ($p=0.165$) (Table 10). The CPT treatments shared similar results, with no significant treatment effect for T_I ($p=0.499$). However, there was a highly significant effect of time and % CO_2 ($p<0.001$), but no significant interaction between treatment (dose) and time and % CO_2 ($p=0.826$) (Table 9). Lastly, there was no significant treatment effect for T_E ($p=0.882$). There was a highly significant effect of time and % CO_2 ($p<0.001$) but no significant interaction between treatment (dose) and time and % CO_2 ($p=0.125$) (Table 11).

Similarly, MIF resulted in the same significance trend between time and CO_2 for both THEO and CPT treatments ($p<0.001$). Looking at THEO, there was no significant treatment effect for MIF ($p=0.270$) and slightly significant interaction between treatment (dose) and time and % CO_2 ($p=0.026$) (Table 12). When multiple comparisons were made between doses of THEO and saline at different times and % CO_2 by the Holm-Sidak method, 40mg/kg of THEO increased MIF at 15 minutes at 5 and 6% CO_2

(p=0.006,0.018), 30 minutes at 5 and 6% CO_2 (p=0.005, 0.026), and 45 minutes at 6% CO_2 (p=0.026) (Fig. 12).

CPT studies did not show a difference between treatments (Fig. 13). There was no significant treatment effect (p=0.879). However, there was a highly significant effect of time and % CO_2 (p<0.001) but no significant interaction between treatment (dose) and time and % CO_2 (p=0.905) (Table 13). A summary of all p-values for each treatment is listed for comparison (Table 14).

Raw values for both THEO and CPT had no significant differences in treatment for V_E, f, V_T, T_I, T_E, and MIF (Figs. 14-25).

Brainstem Histology

When comparing the brainstems of the 4-day-old rat to the 7-day-old rat, there are two distinct differences. The younger, 4-day-old rat brain section contained more folds within the brain and had less nerve and neuroglial cells (Fig. 26). In contrast, the older, 7-day-old rat brain section had lost those folds (Fig. 27) and gained more numerous nerve and neuroglial cells (Figs. 28-30).

DISCUSSION

Respiration Studies

Theophylline studies revealed that 40mg/kg was the drug dose with the most reactivity, as shown by its significant increases in V_E, V_T, and MIF (Tables 2, 6, 12). These increases in V_E, V_T, and MIF are all consistent with a similar study by Bleul *et. al.* (2010) involving neonatal calves. They were given injections of THEO with the intention of maintaining regular respiration, improving oxygen uptake, and carbon dioxide release. Although it took over an hour to show increases in respiration, the effects were observed. The long wait-time could have been because they used a low dose of THEO. In my study, the highest dose of THEO was effective, but only at the highest concentrations of inhaled of CO_2. For instance, 40mg/kg of THEO was only reactive at high CO_2 percentages (5-6%), but did not show a significant trend in time after injection of the drug since there were increases in V_E, V_T, and MIF at 15 minutes, 30 minutes, and 45 minutes (Figs. 2, 6, 12). The other, lower doses of THEO (10, 20 mg/kg) did not result in increases in these parameters (V_E, V_T, and MIF), so using those doses would not be recommended, even though they showed signs of significance at certain times. Another study by Milic-Emili & Grunstein (1976) supports the results from this study that found that respiration (V_E and V_T) increased at higher CO_2 percentages. They experimented with different levels of CO_2 on cat respiration and found that increasing the amount of CO_2 into the body, increases respiration, specifically V_E and V_T (CO_2 response curve).

The other parameters, T_I and T_E, did not increase when any dose of THEO was added (Figs, 8 and 10), and no significant CO_2 response trend existed for respiration rate

(Fig. 4). My results supported the previous work by Bleul *et. al.* (2010) and Rigatto *et. al.* (1988), where they found that breathing rate (f) remained constant no matter what treatment was added. T_I and T_E did have significant CO_2 response curves with T_I increasing while T_E decreased, but there was no effect of THEO treatment, which is logical since the breathing rate had no pattern.

In addition to THEO, CPT is considered a more efficient alternative due to its increased potency and affinity for adenosine A_1 receptors. CPT is developed by the addition of a cycloalkyl group to the 8 position of theophylline. This small change allows CPT to be given at a much lower dose than THEO, while exhibiting the same effects of increased depth and consistency of respiration and lowering the risk of harmful side effects that higher doses can cause (Shamin 1988). Previous studies on the effects of CPT on respiration in neonatal rats by Albers & McGilliard (2012) showed that 640 μg/kg and 1280 μg/kg CPT significantly increased respiratory parameters including V_E, V_T, and MIF. These doses, although high for CPT, are in micrograms, while an equivalent dose of THEO would have to be in milligrams to have any significant effect, which supports the idea of CPT being a more potent and efficient drug than THEO.

However, in my study, CPT had no doses that were significantly different from saline, which is surprising since we expected CPT be more potent than THEO. Specifically, CPT has a higher affinity for A_1 and A_2 receptors than THEO, which means that it should more easily bind to these receptors. One reason for CPT's ineffectiveness in my study could be the doses administered. The 3 doses of CPT (320 μg/kg, 640 μg/kg, and 1280 μg/kg) were much smaller than the THEO doses (10 mg/kg, 20 mg/kg, 40 mg/kg), and rightfully so since they should have an increased potency, but even then, the

CPT dose could be larger. One problem with increasing the dose is that solubility is extremely low. Other solvents have to be explored to keep the drug in solution. At the doses tested, the null hypothesis could not be rejected, despite previous work showing that 640 ug/kg, and 1280 ug/kg CPT produced significant increases in respiration (Albers & McGilliard, 2012). For future studies it would be important to insure the drug being used is new and made correctly, as well as increasing the dose of CPT. If solubility were a problem, the solvent could be changed to allow delivery of higher doses. A higher dose could also be produced through intravenous infusion of CPT, but this would be technically very difficult in a newborn rat.

When comparing THEO to CPT, THEO is the apparent choice to increase respiration in neonates because it was the only drug to show any significant increase in the respiratory parameters. This is consistent with other studies claiming that theophylline remains a widely used drug to treat neonates. Durand *et.al.* (1987) used THEO paired with CO_2 to wean premature infants off of a ventilator. They found that treatment with THEO benefitted respiration without a ventilator by increasing bronchodilation and increasing diaphragm contractility. Specifically, compared to control infants, those given THEO were on the breathing machine for less time, did not require reintubation, and had overall more consistent breathing patterns.

Brainstem Histology

These observations of increased respiration with the administration of methylxanthines paired with CO_2 are effective in preterm infants. These drugs are required because the brain (along with respiratory organs) are not as developed in the younger infant. In a dissection of a 4 and 7-day-old rat brain, there was a visible

difference in brain development. The 4-day-old rat brain had large folds or folia, while the 7-day-old rat brain lost those folds and was much denser in neuroglial and nerve cells. These differences in the two brains are plausible since as the rat matures, the brain also matures (Snider *et. al.*1950; Casey *et. al.* 2005). The folds disappear as the brain grows, and the new cells are synthesized as a part of this growth and development as well (Snider *et. al.*1950; Casey *et. al.* 2005). Although age relationships weren't defined for this study, it is still important to note the apparent growth of the brain in just four days and how this growth along with treatment of THEO/CPT and CO_2 significantly increases respiration.

Other possibilities for future studies would be to observe long-term effects of methylxanthines like THEO and CPT. The use of caffeine (1, 3, 7-trimethylxanthine) in preterm infants has shown that, in the short term, the rate of bronchopulmonary dysplasia, which is defined as the need for supplemental oxygen after 36 weeks of living is reduced (Schmidt *et. al.* 2006). In addition, infant reliance on supplemental oxygen from respirators was greatly reduced (Schmidt *et. al.* 2007). In the long term, methylxanthines showed reduced neurodevelopmental disabilities such as cerebral palsy, cognitive delay, and hearing loss (Schmidt *et. al.* 2012). Similarly, methylxanthines have been shown to reduce an infant's ability to gain weight in the first two weeks of treatment, but following this short weight loss, weight gain continues normally (Schmidt *et. al.* 2006).

CONCLUSIONS

Premature infants plagued with respiration problems like periodic breathing and apnea can be treated with respiratory stimulants like THEO and CPT coupled with CO_2. Although there was not an overall significant difference between doses of THEO, the highest dose, 40mg/kg, showed significant increases in minute ventilation, tidal volume, and mean inspiratory flow when paired with 5-6% CO_2. The dose of 10 mg/kg of THEO also showed increases in minute ventilation and tidal volume at the higher CO_2 percentages. In contrast, CPT showed no significant increases in respiration at any dose. For both CPT and THEO, CO_2 exposure produced a consistent increase in minute ventilation, tidal volume, and mean inspiratory flow, but not in respiratory rate all of which were independent of the drug response. Consequently, CO_2 alone produced increases in minute ventilation, tidal volume, and mean inspiratory flow, and when paired with a dose of 40 mg/kg of THEO, these parameters increased further showing that the highest doses of THEO paired with the highest doses of CO_2 produce a significant increase in respiration. From my findings, I would suggest that preterm infants suffering from respiration problems should be put on THEO paired with 5% CO_2. Although further experimentation should be done with similar drugs and different entry routes into the body, THEO would be a good treatment to aid a premature infant with breathing problems.

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Table 1: Dehydration solutions using various amounts of t-butyl alcohol

	Solution I	Solution II	Solution III	Solution IV	Solution V
% Alcohol	70	85	100	100	100
Distilled Water	30 ml	15 ml	--	--	--
100% ethanol	50 ml	50 ml	45 ml	25 ml	--
t-butyl alcohol	20 ml	35 ml	55 ml	75 ml	100 ml

Table 2: Two way repeated measures ANOVA of Theophylline for Minute Ventilation.

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Treatment (THEO)	3	10284	3428	0.754	0.529
Rat (Treatment)	28	127310	4546		
Time $\%CO_2$	23	115698	5030	48.172	<0.001
Treatment x Time $\%CO_2$	69	11120	161	1.543	0.004
Residual	644	67249	104		
Total	767	331663	432		

Table 3: Two way repeated measures ANOVA of Cyclopentyltheophylline for Minute Ventilation.

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Treatment (CPT)	3	7301	2433	1.824	0.166
Rat (Treatment)	28	37355	1334		
Time $\%CO_2$	23	86592	3764	92.693	<0.001
Treatment x Time $\%CO_2$	69	2521	36	0.900	0.703
Residual	644	26157	40		
Total	767	159928	208		

Table 4: Two way repeated measures ANOVA of Theophylline for Respiratory Rate.

Source of Variation	DF	SS	MS	F	P
Treatment (THEO)	3	16939	5646	1.140	0.350
Rat (Treatment)	28	138668	4952		
Time $\%CO_2$	23	4298	186	1.422	0.092
Treatment x Time $\%CO_2$	69	10571	153	1.166	0.178
Residual	644	84635	131		
Total	767	255113	332		

Table 5: Two way repeated measures ANOVA of Cyclopentyltheophylline for Respiratory Rate.

Source of Variation	DF	SS	MS	F	P
Treatment (CPT)	3	1281	427	0.127	0.943
Rat (Treatment)	28	94023	3357		
Time $\%CO_2$	23	8311	361	3.112	<0.001
Treatment x Time $\%CO_2$	69	7774	112	0.970	0.548
Residual	644	74787	116		
Total	767	186177	2412		

Table 6: Two way repeated measures ANOVA of Theophylline for Tidal Volume.

Source of Variation	DF	SS	MS	F	P
Treatment (THEO)	3	46139	15379	5.166	0.006
Rat (Treatment)	28	83365	2977		
Time $\%CO_2$	23	120729	5249	33.710	<0.001
Treatment x Time $\%CO_2$	69	11731	170	1.092	0.293
Residual	644	100279	155		
Total	767	362244	472		

Table 7: Two way repeated measures ANOVA of Cyclopentyltheophylline for Tidal Volume.

Source of Variation	DF	SS	MS	F	P
Treatment (CPT)	3	4376	1458	0.280	0.840
Rat (Treatment)	28	146050	5216		
Time $\%CO_2$	23	66750	2902	22.858	<0.001
Treatment x Time $\%CO_2$	69	9198	133	1.050	0.374
Residual	644	81767	126		
Total	767	308143	401		

Table 8: Two way repeated measures ANOVA of Theophylline for Time of Inspiration.

Source of Variation	DF	SS	MS	F	P
Treatment (THEO)	3	66903	22301	0.359	0.783
Rat (Treatment)	28	1739724	62133		
Time $\%CO_2$	23	79957	3476	10.311	<0.001
Treatment x Time $\%CO_2$	69	19843	287	0.853	0.794
Residual	644	217133	337		
Total	767	2123562	2768		

Table 9: Two way repeated measures ANOVA of Cyclopentyltheophylline for Time of Inspiration.

Source of Variation	DF	SS	MS	F	P
Treatment (CPT)	3	418859	139619	0.810	0.499
Rat (Treatment)	28	4823544	172269		
Time $\%CO_2$	23	173027	7522	6.356	<0.001
Treatment x Time $\%CO_2$	69	68119	987	0.834	0.826
Residual	644	762269	1183		
Total	767	6245820	8143		

Table 10: Two way repeated measures ANOVA of Theophylline for Time of Expiration.

Source of Variation	DF	SS	MS	F	P
Treatment (THEO)	3	34455	11485	0.687	0.567
Residual	28	467765	16705		
Time $\%CO_2$	23	21662	941	2.530	<0.001
Treatment x Time $\%CO_2$	69	30214	437	1.176	0.165
Residual	644	239479	372		
Total	767	793846	1035		

Table 11: Two way repeated measures ANOVA of Cyclopentyltheophylline for Time of Expiration.

Source of Variation	DF	SS	MS	F	P
Treatment (CPT)	3	7840	2613	0.220	0.882
Rat (Treatment)	28	332533	11876		
Time $\%CO_2$	23	46549	2023	7.886	<0.001
Treatment x Time $\%CO_2$	69	21522	311	1.212	0.125
Residual	644	165705	257		
Total	767	574151	748		

Table 12: Two way repeated measures ANOVA of Theophylline for Mean Inspiratory Flow.

Source of Variation	DF	SS	MS	F	P
Treatment (THEO)	3	40099	13366	1.377	0.270
Rat (Treatment)	28	271741	9705		
Time $\%CO_2$	23	13527	588	3.485	<0.001
Treatment x Time $\%CO_2$	69	16120	233	1.384	0.026
Residual	644	108690	168		
Total	767	450179	586		

Table 13: Two way repeated measures ANOVA of Cyclopentyltheophylline for Mean Inspiratory Flow.

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Treatment (THEO)	3	7519	2506	0.223	0.879
Rat (Treatment)	28	314345	11226		
Time $\%CO_2$	23	47550	2206	8.172	0.032
Treatment x Time $\%CO_2$	69	6793	98	0.777	0.905
Residual	644	81695	126		
Total	767	415014	541		

Table 14: P values for Theophylline and CPT studies.

		% Change VE	% Change F	% Change VT	% Change TI	% Change TE	% Change MIF
Theophylline Studies	P value for Treatment	0.529	0.350	0.006	0.783	0.567	0.270
	P value for Time$\%CO_2$	<0.001	0.092	<0.001	<0.001	<0.001	<0.001
	P value Treatment xTime$\%CO_2$	0.004	0.178	0.293	0.794	0.165	0.026
CPT Studies	P value for Treatment	0.166	0.943	0.840	0.499	0.882	0.879
	P value for Time$\%CO_2$	<0.001	<0.001	<0.001	<0.001	<0.001	0.032
	P value Treatment xTime$\%CO_2$	0.703	0.548	0.374	0.826	0.125	0.905

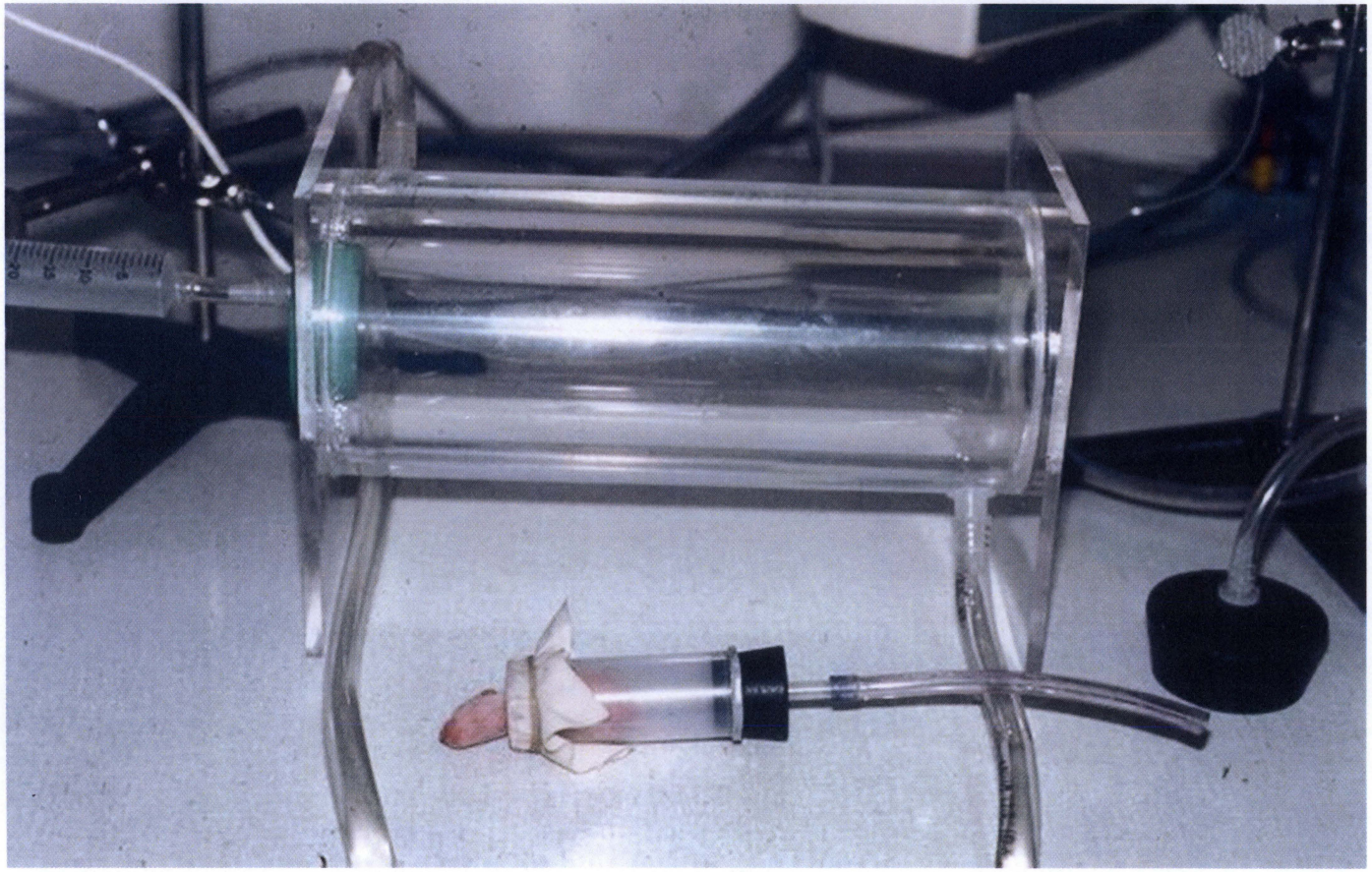

Fig. 1. Rat enclosed in the plethysmograph with environment stabilizing apparatus above.

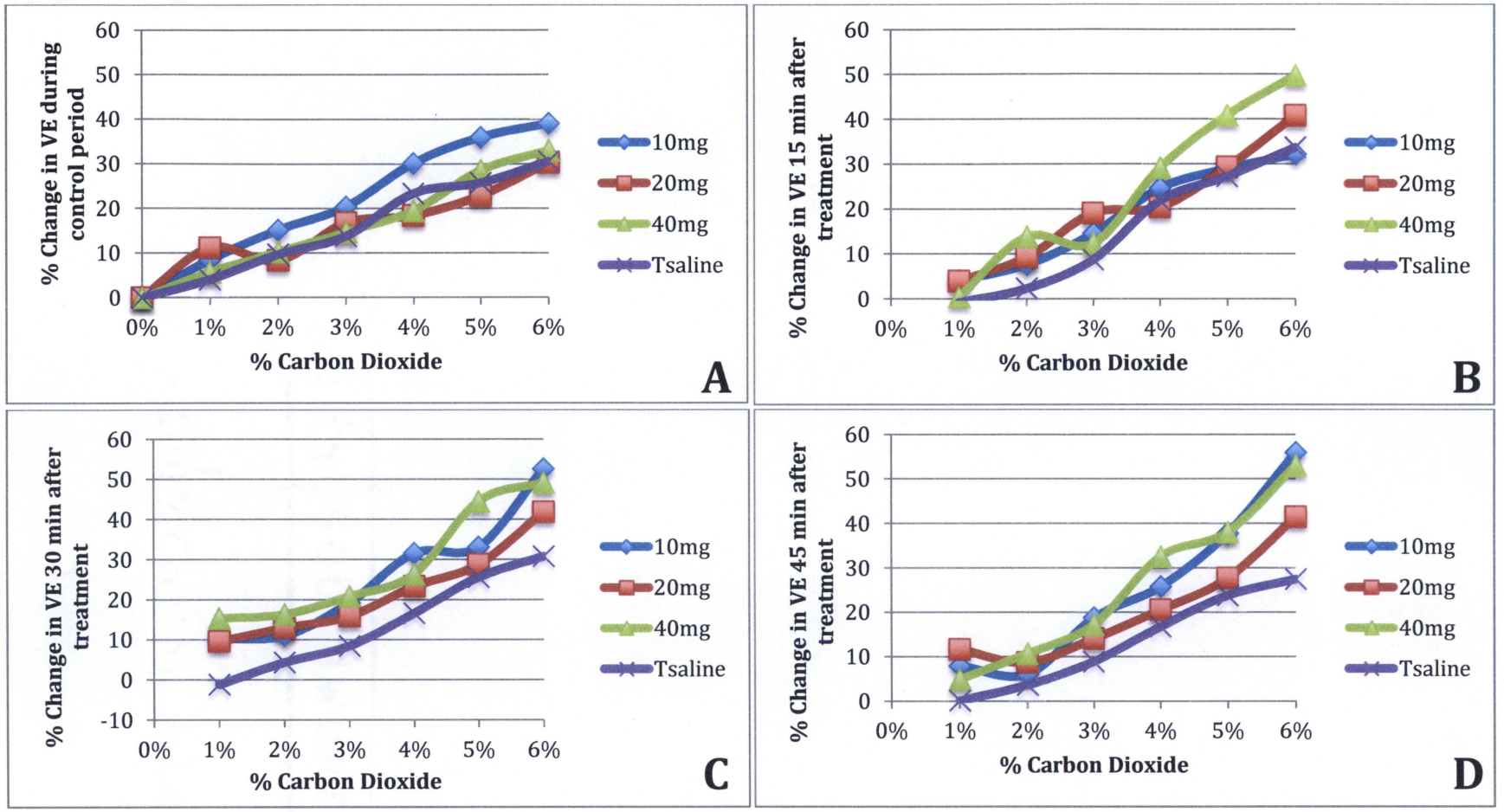

Fig. 2. The effect of THEO on the percent change in minute ventilation response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection.

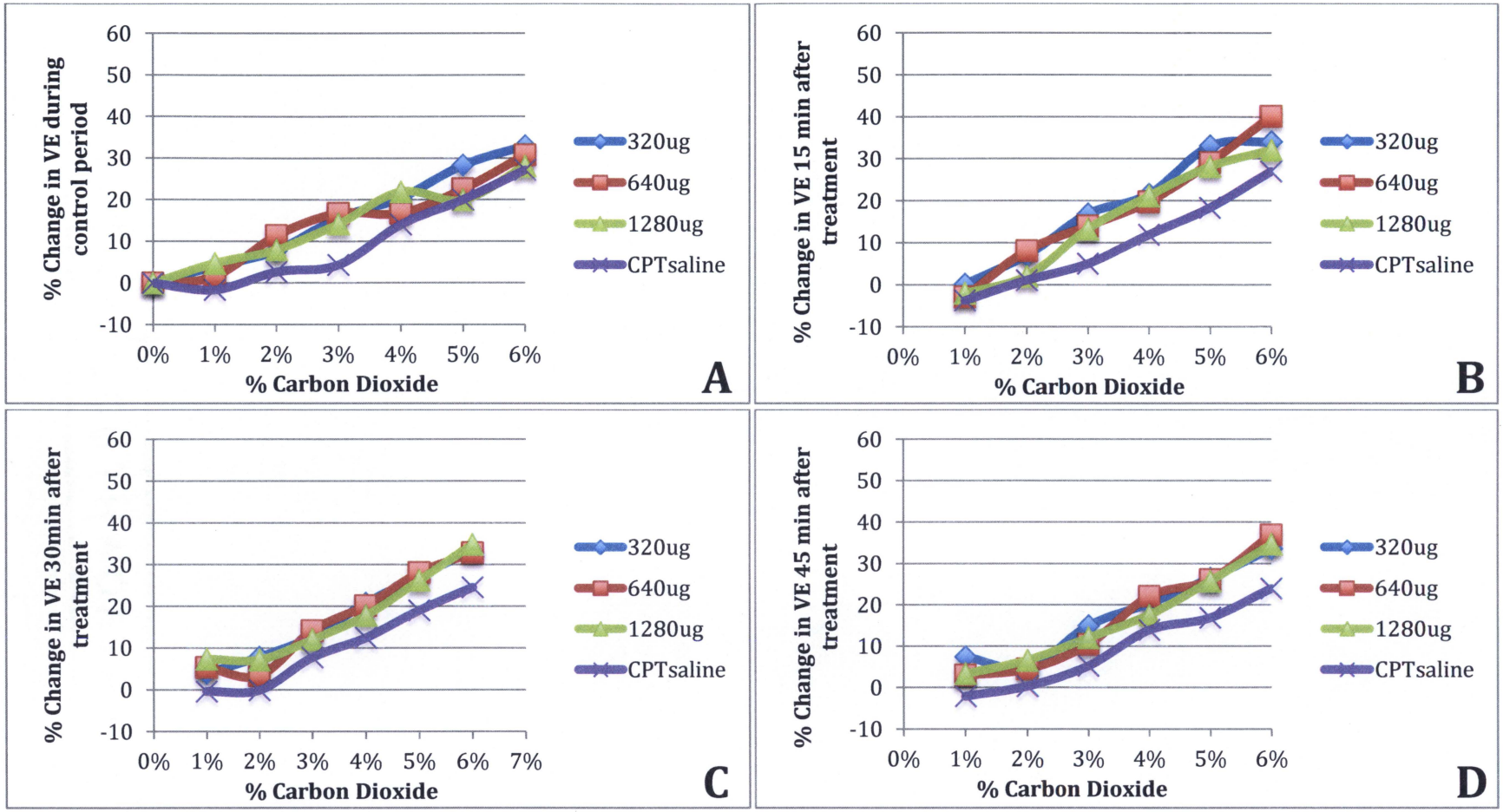

Fig. 3. The effect of CPT on the percent change in minute ventilation response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection.

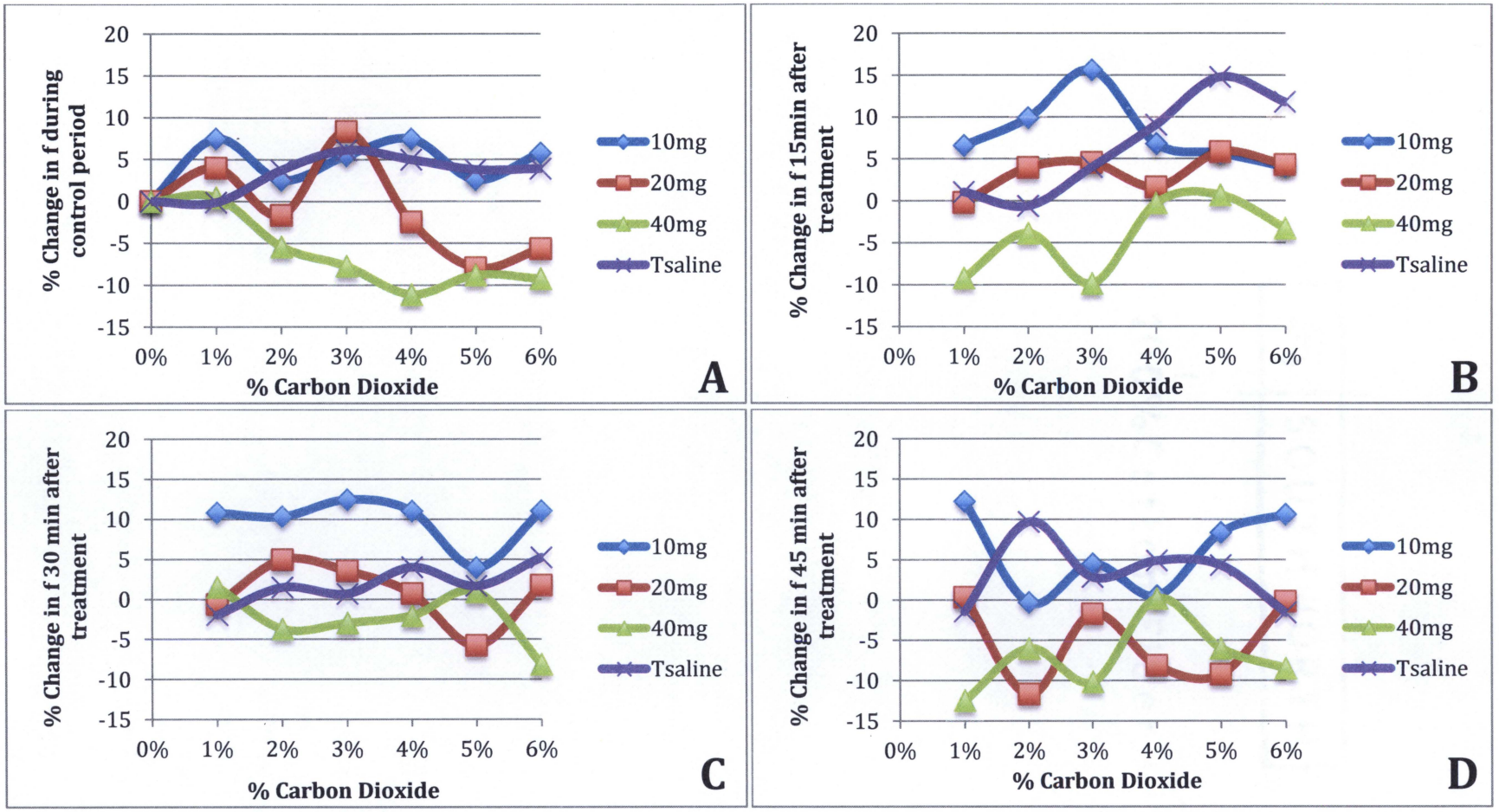

Fig. 4. The effect of THEO on the percent change in respiratory rate response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection.

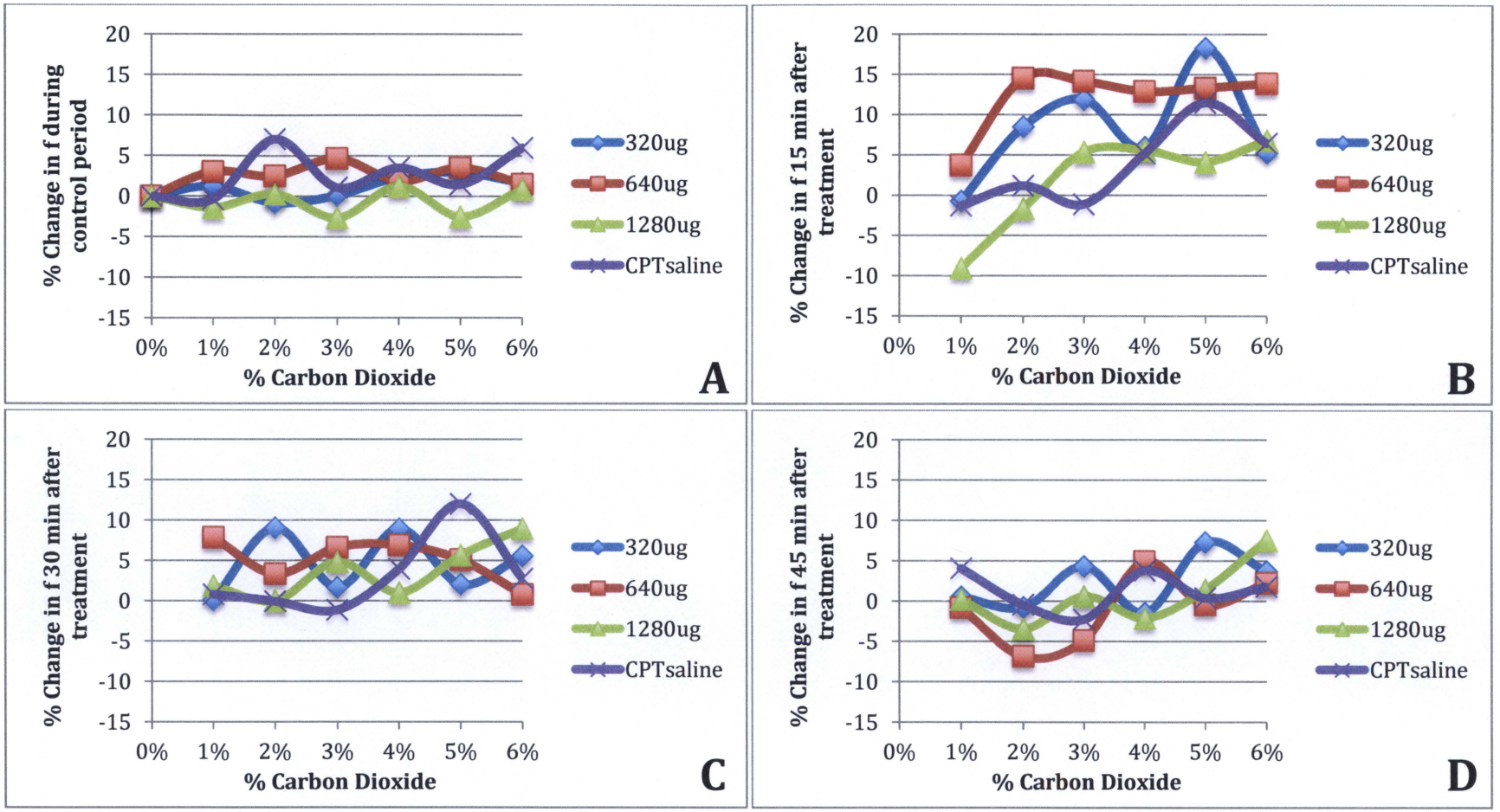

Fig. 5. The effect of CPT on the percent change in respiratory rate response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection.

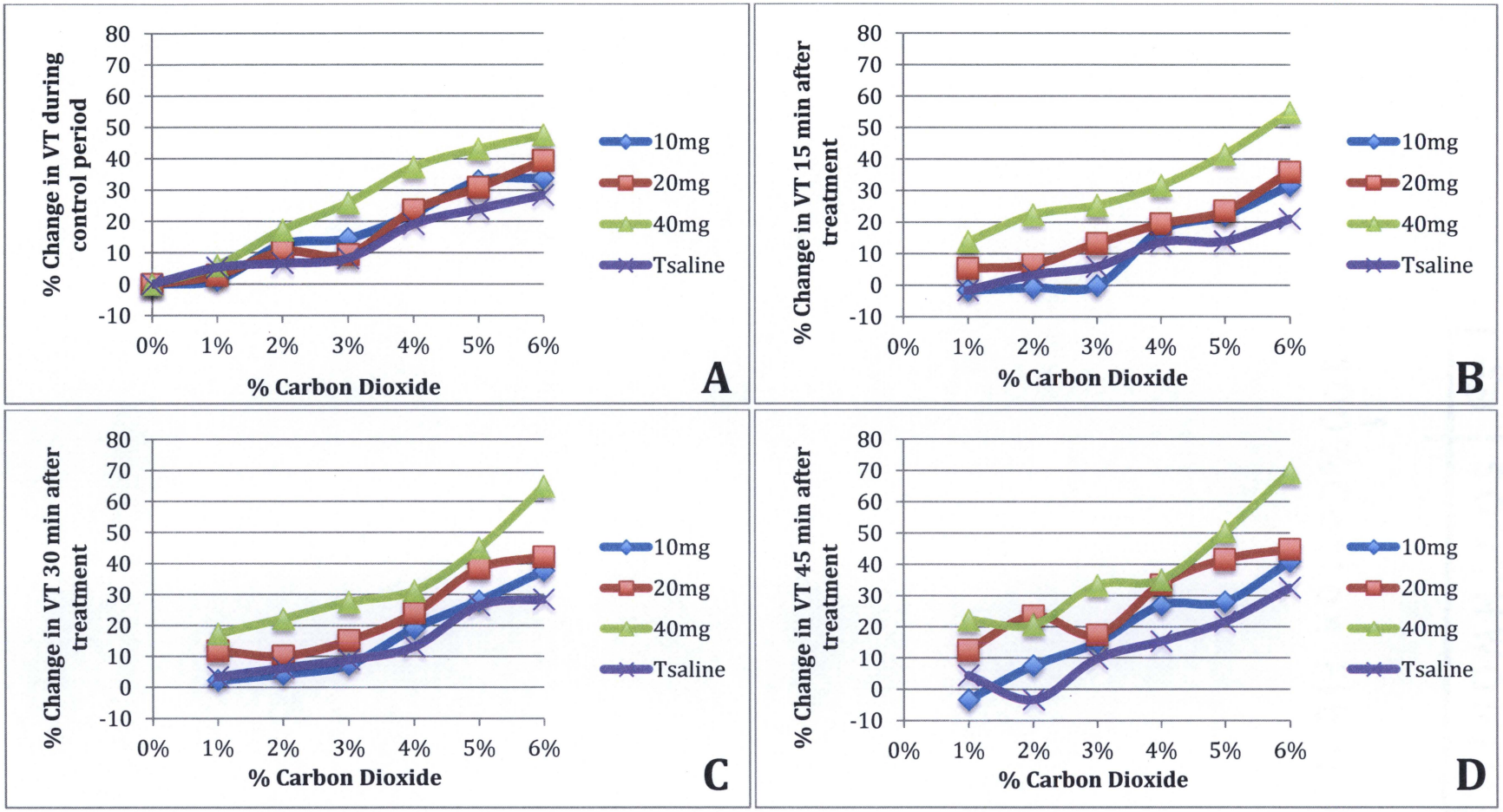

Fig. 6. The effect of THEO on the percent change in tidal volume response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection.

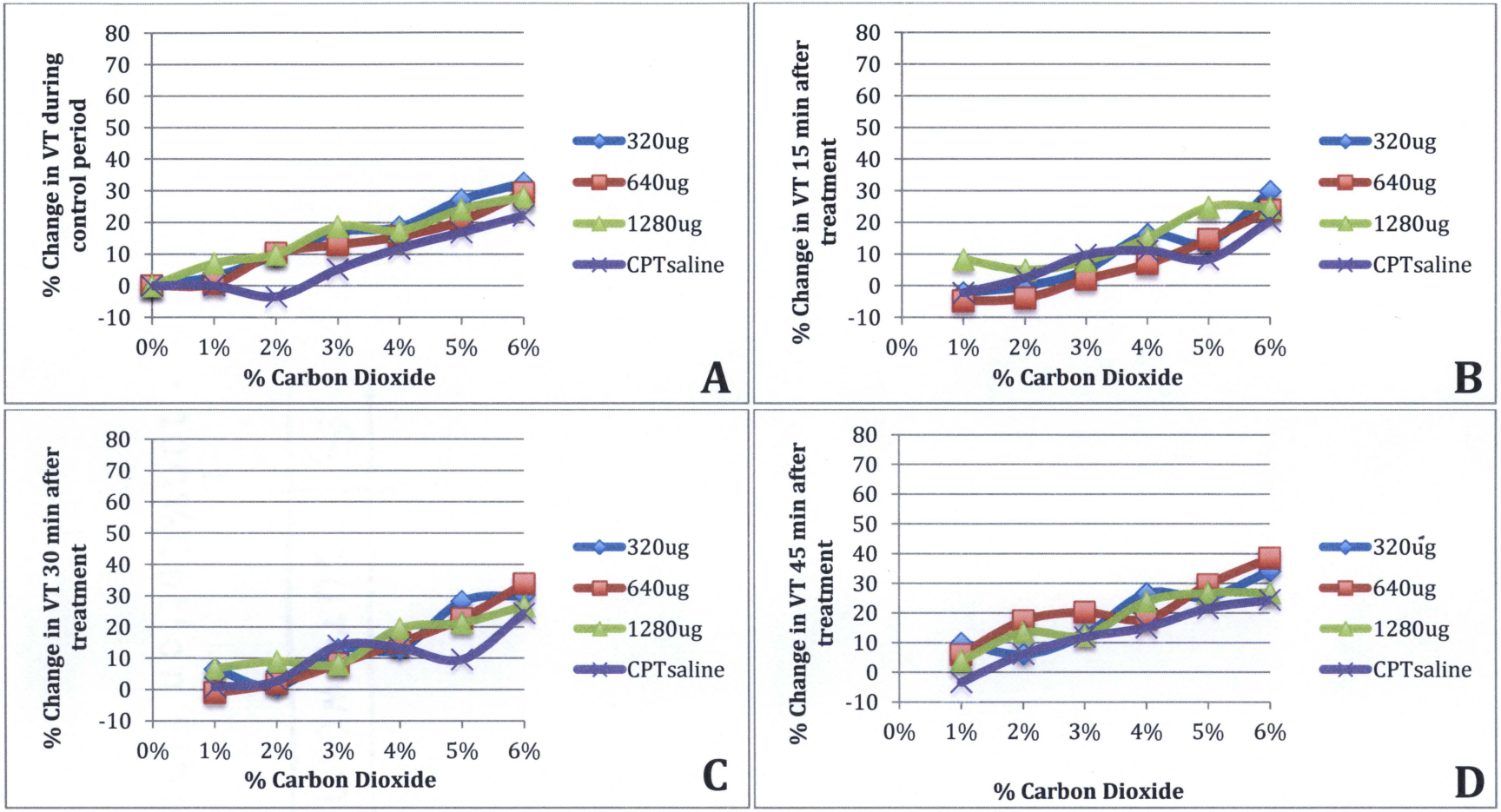

Fig. 7. The effect of CPT on the percent change in tidal volume response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection.

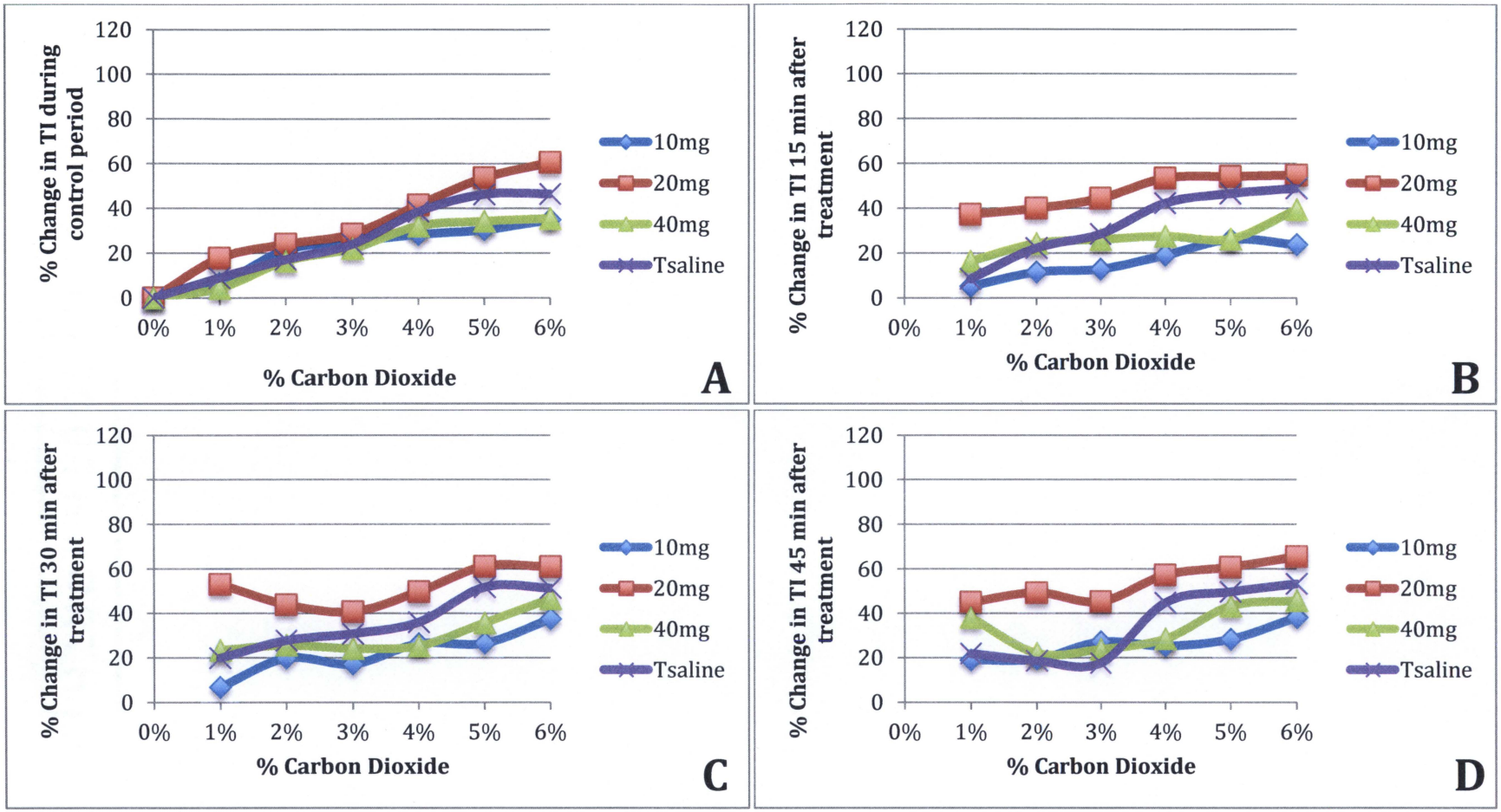

Fig. 8. The effect of THEO on the percent change in time of inspiration response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection.

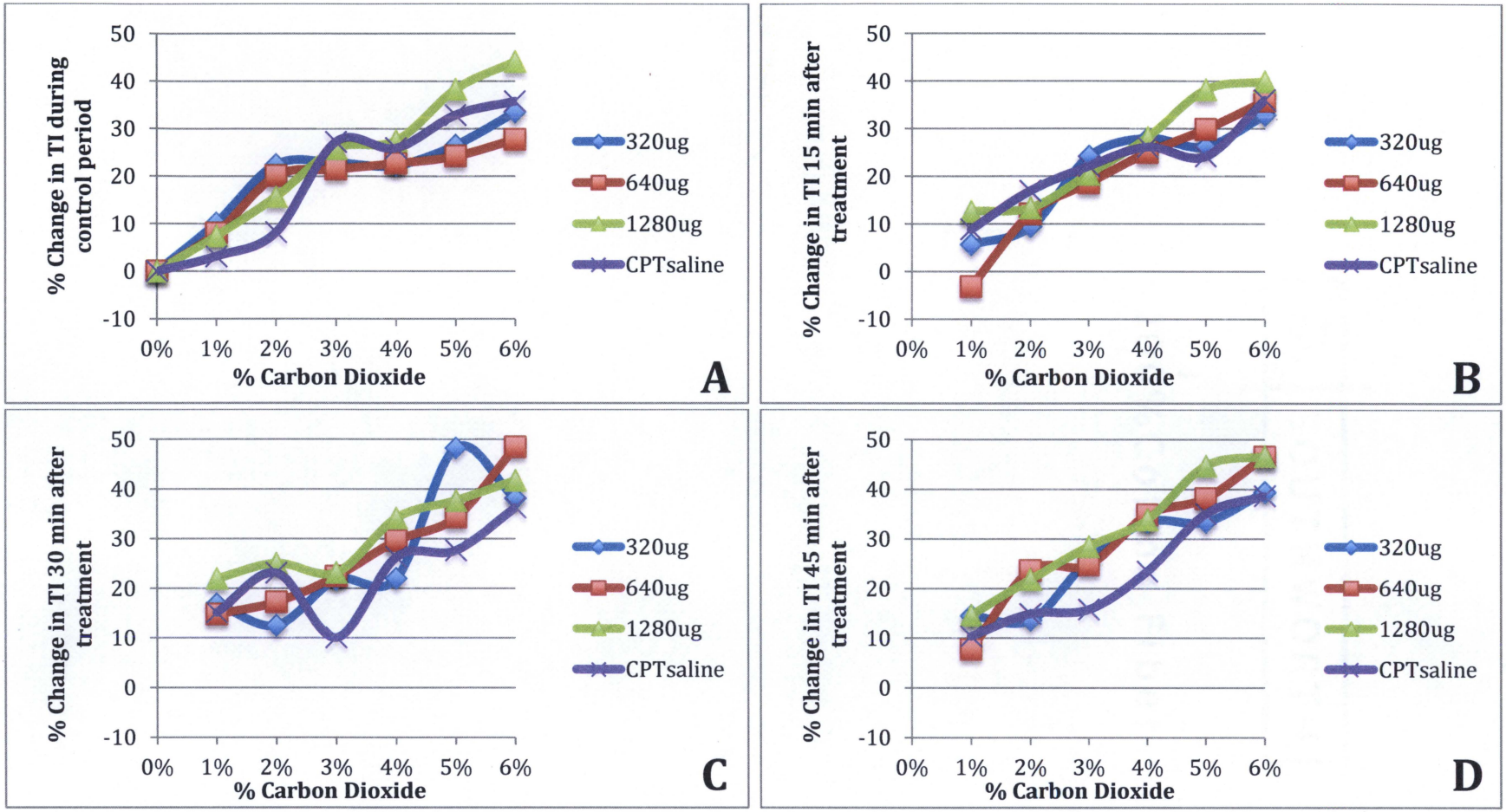

Fig. 9. The effect of CPT on the percent change in time of inspiration response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection.

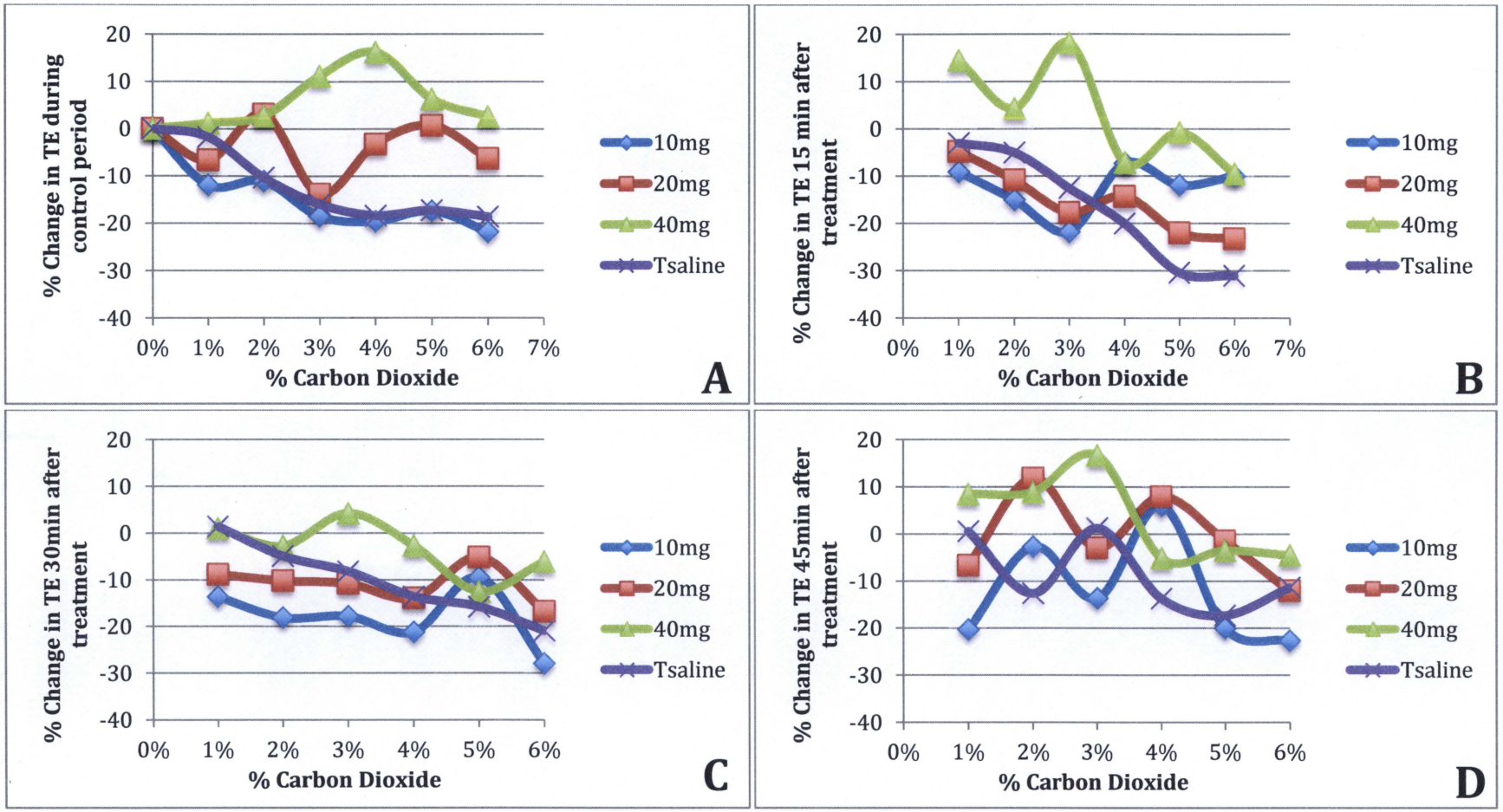

Fig. 10. The effect of THEO on the percent change in time of expiration response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection.

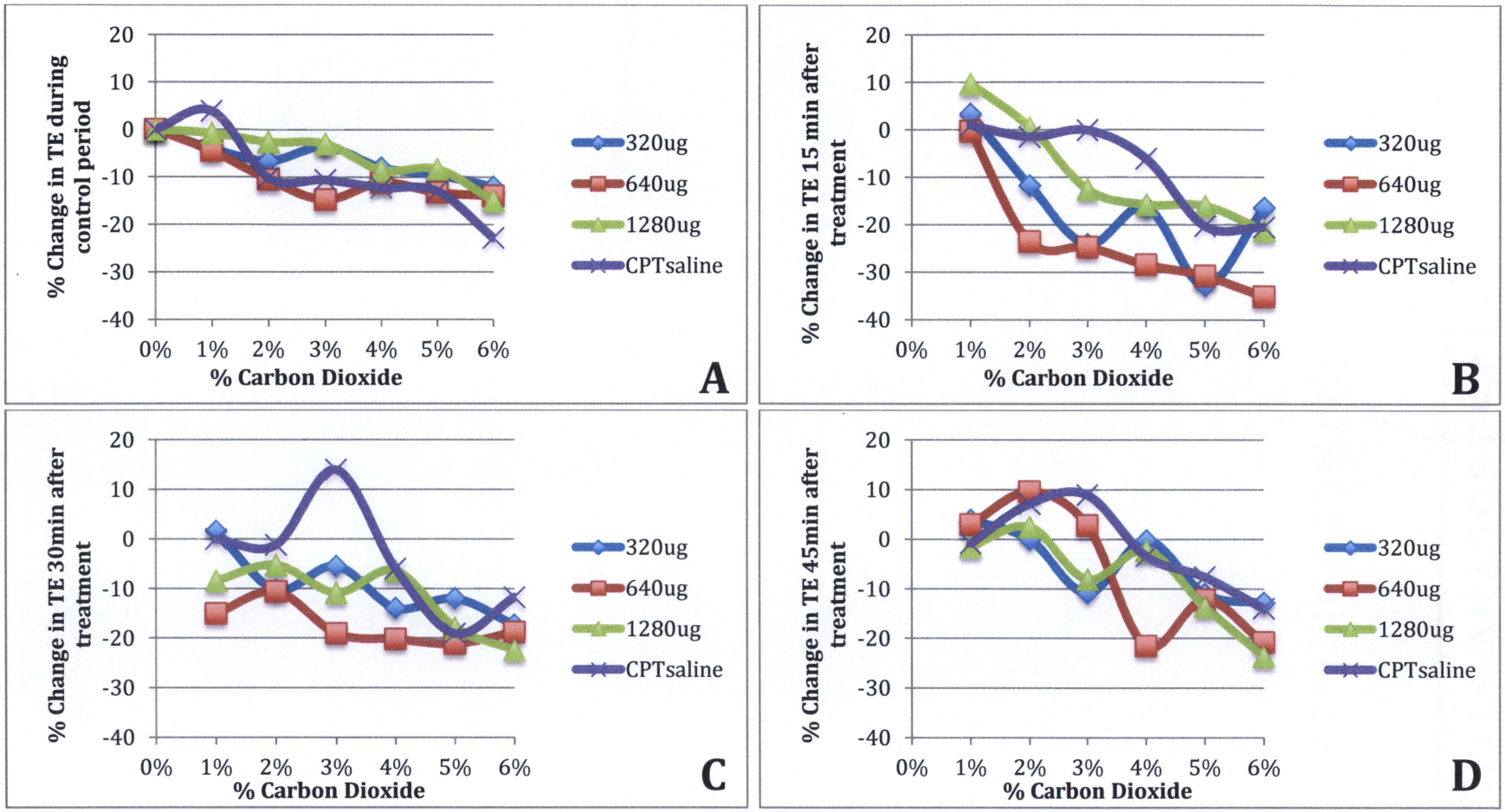

Fig. 11. The effect of CPT on the percent change in time of expiration response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection.

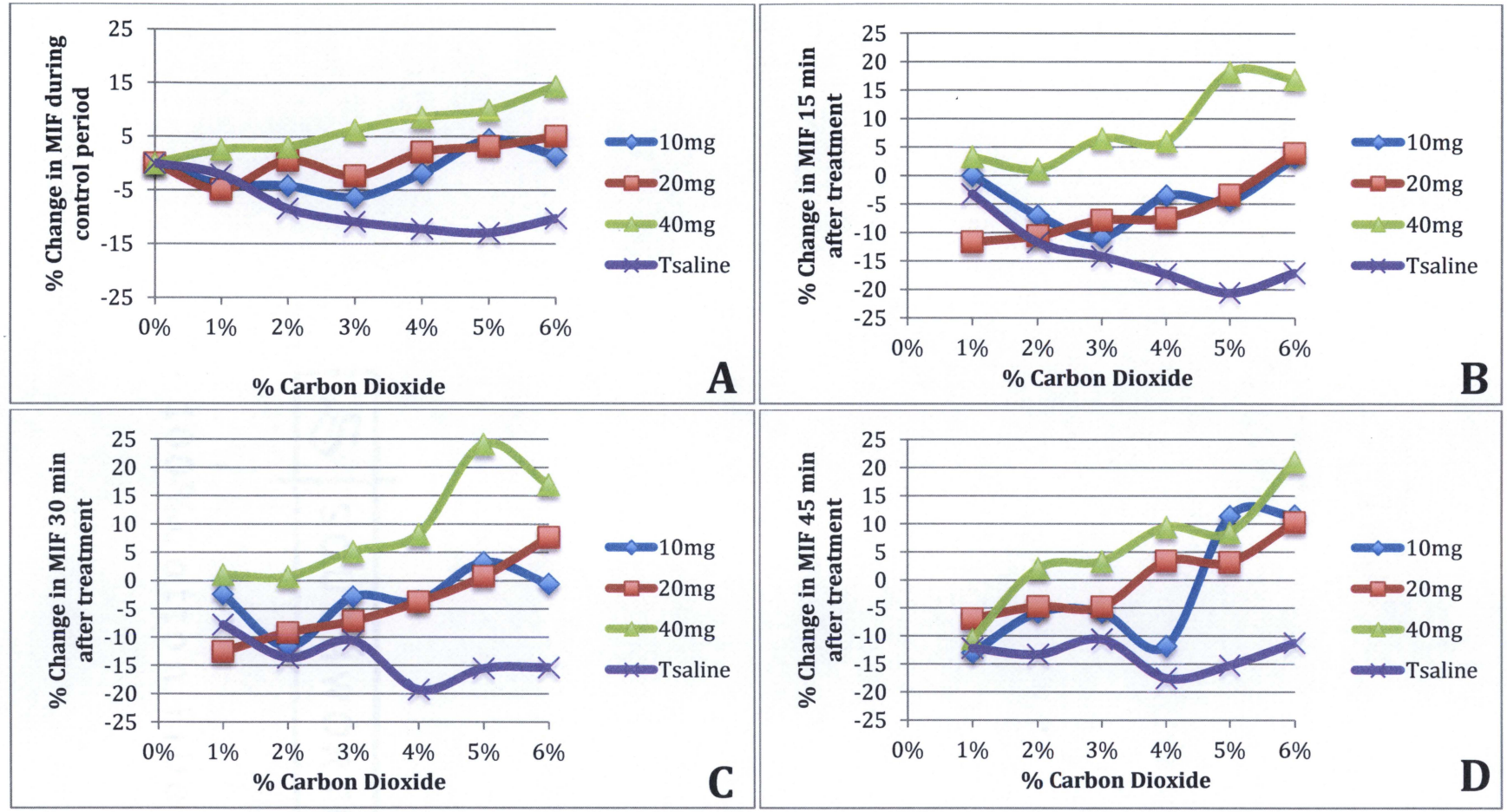

Fig. 12. The effect of THEO on the percent change in mean inspiratory flow response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection.

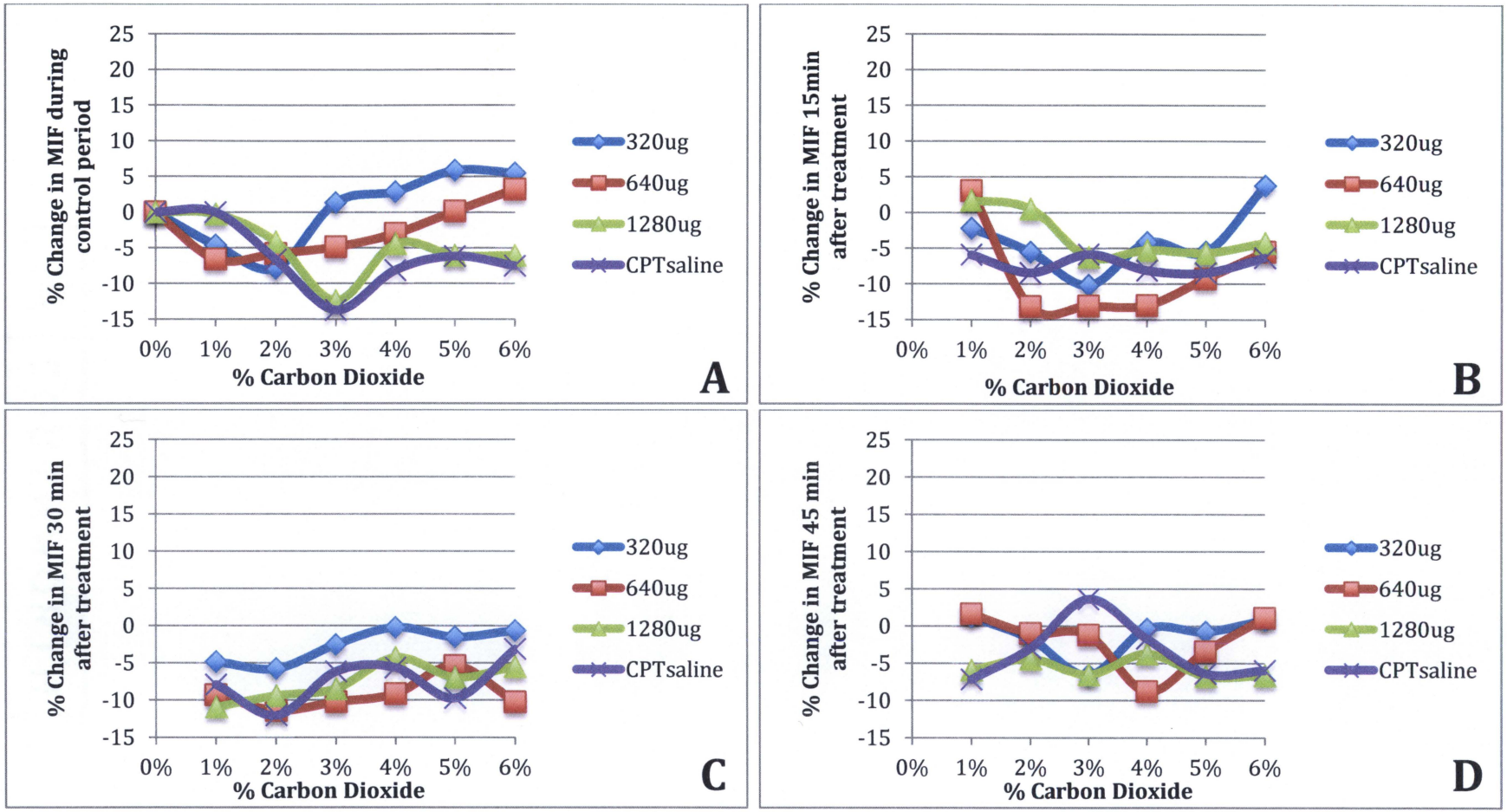

Fig. 13. The effect of CPT on the percent change in mean inspiratory flow response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection.

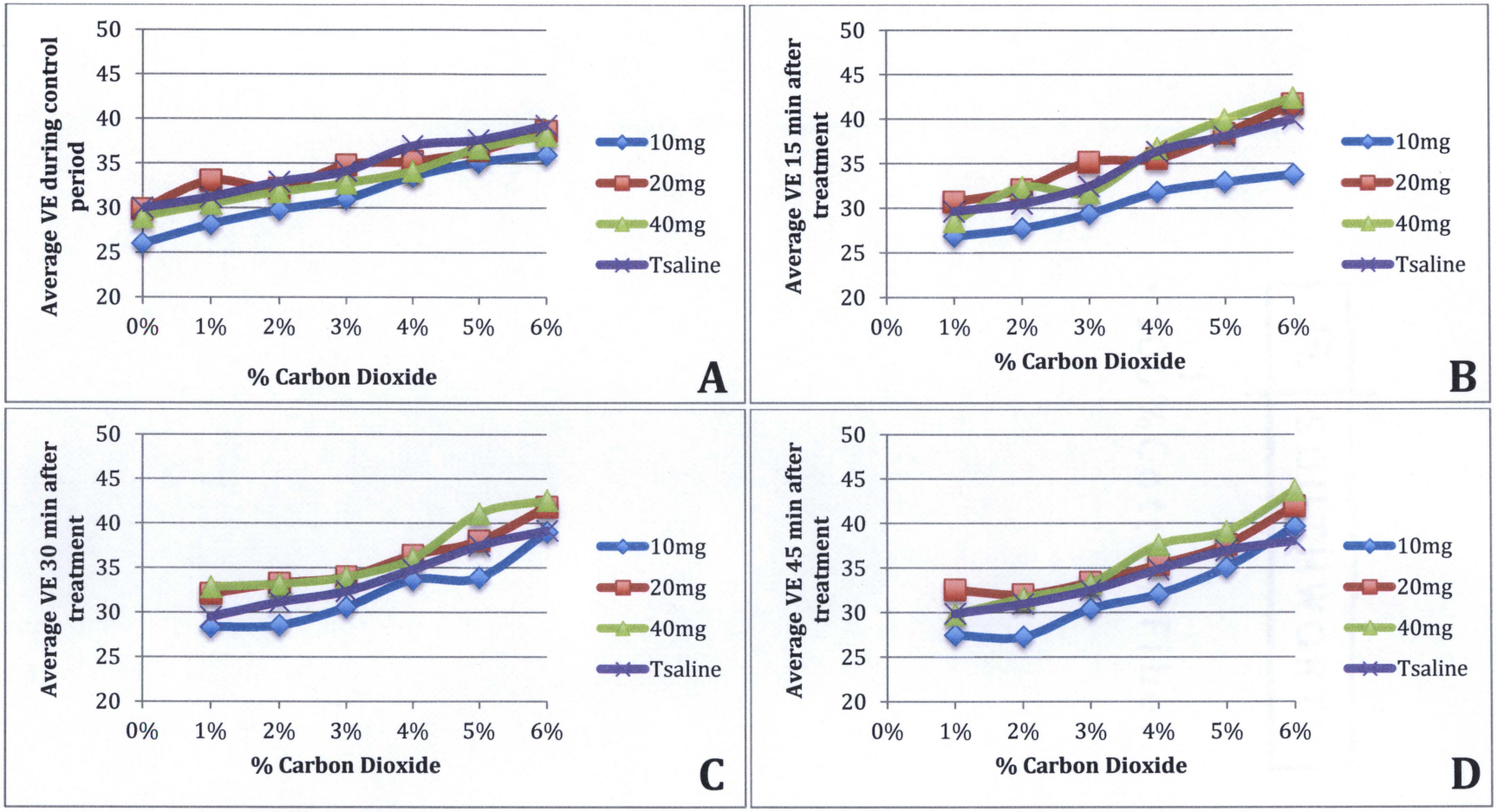

Fig. 14. The effect of THEO on the average minute ventilation response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection.

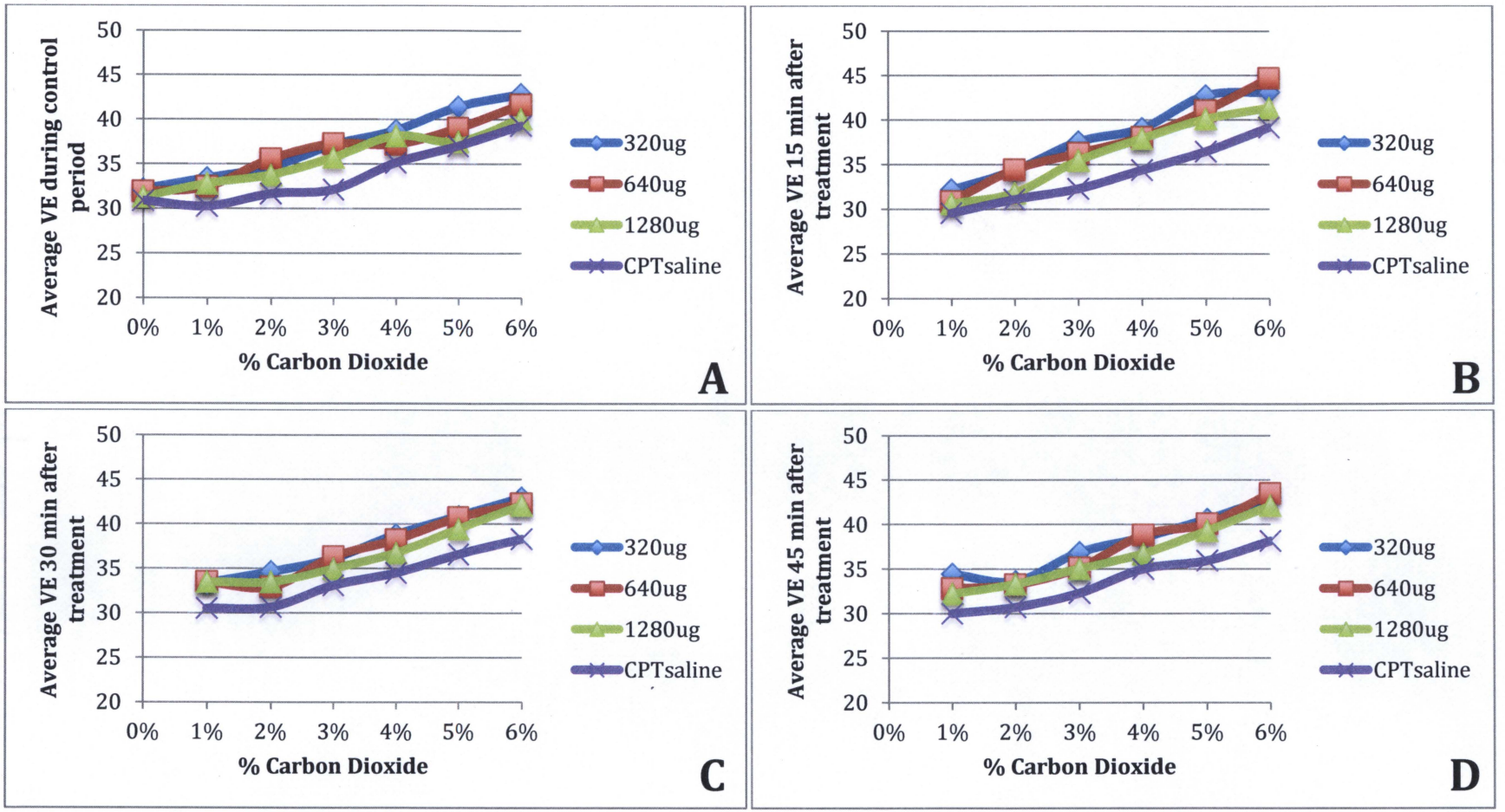

Fig. 15. The effect of CPT on the average minute ventilation response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection.

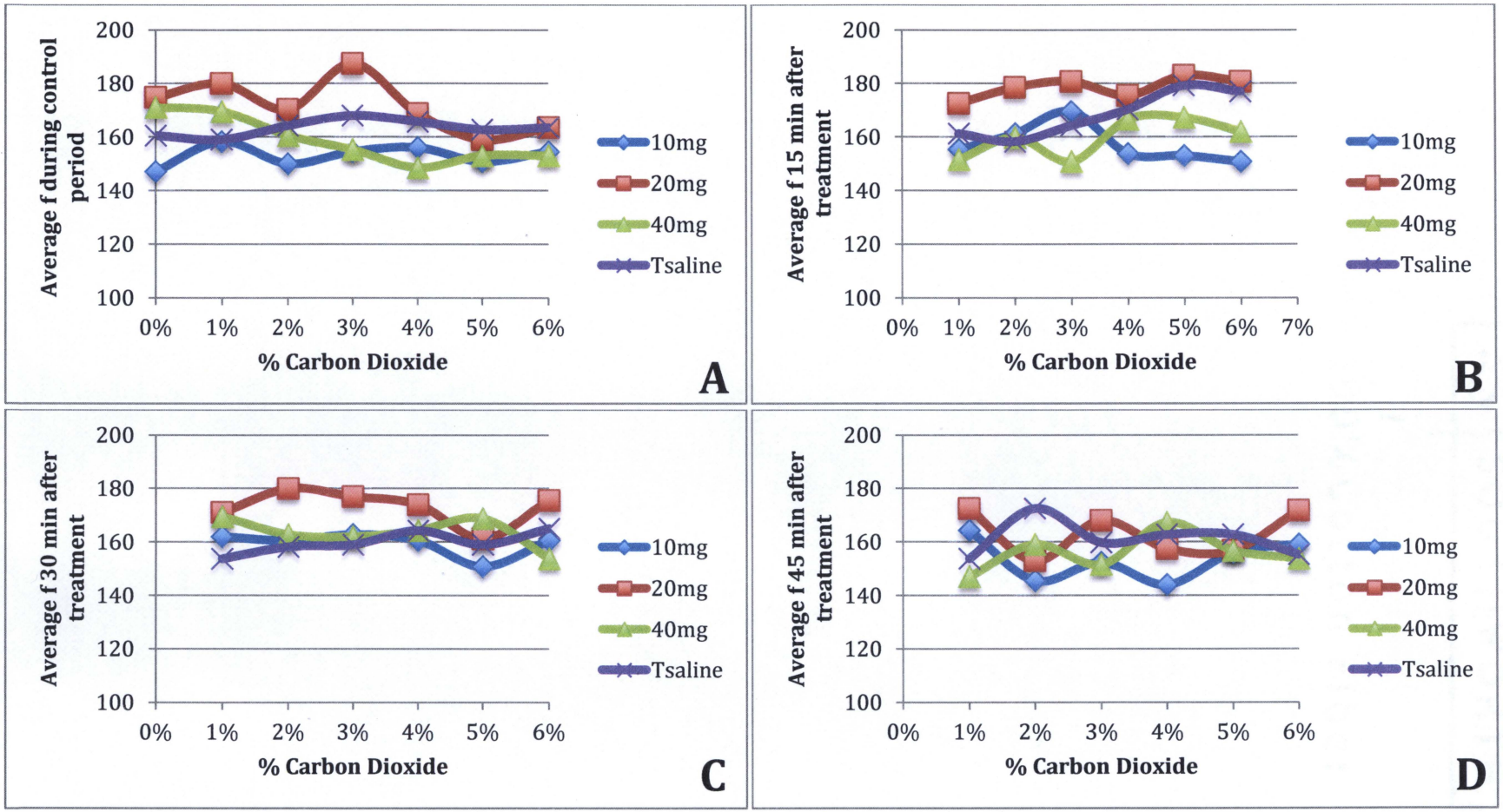

Fig. 16. The effect of THEO on the average respiratory rate response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection.

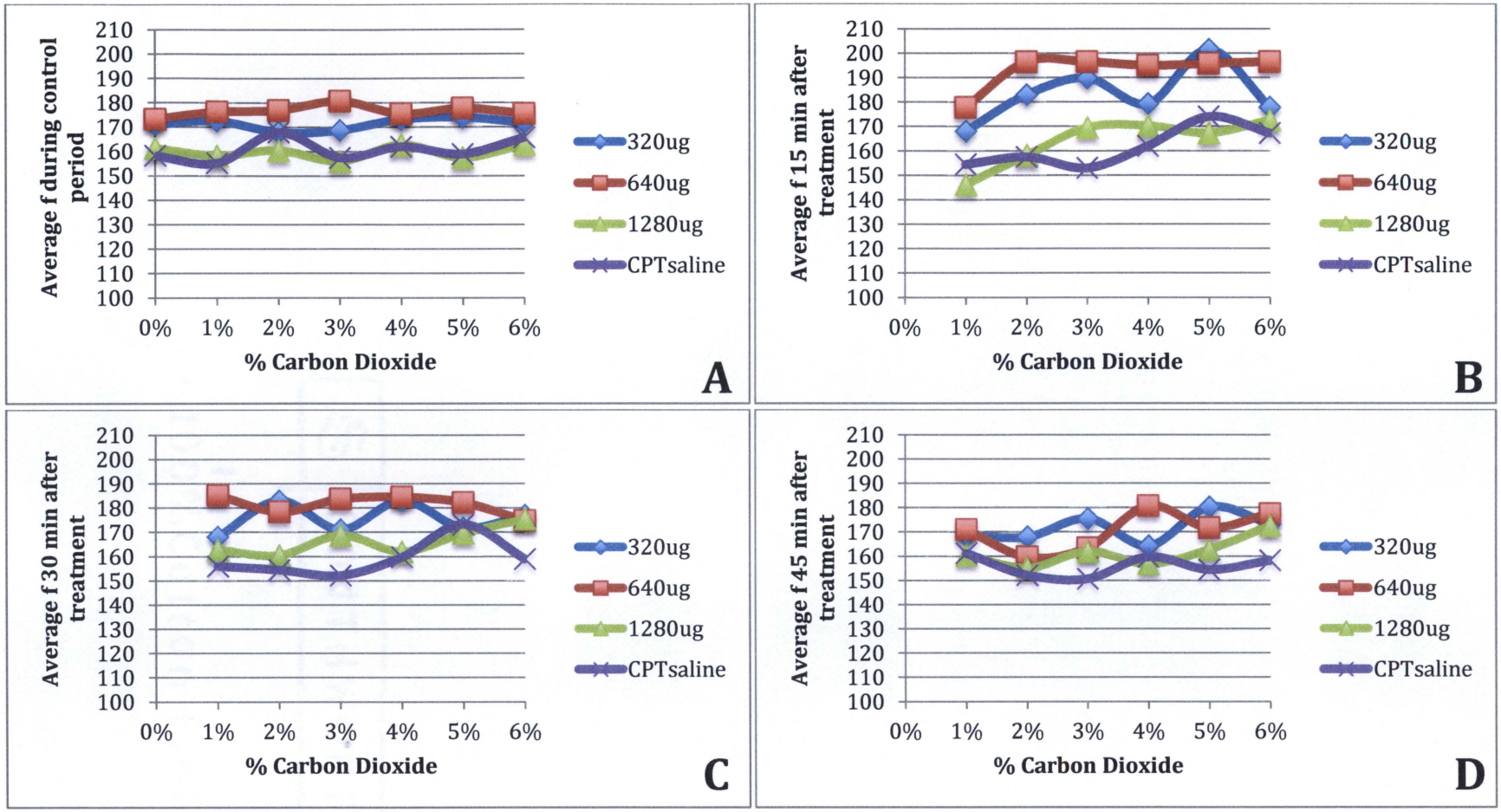

Fig. 17. The effect of CPT on the average respiratory rate response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection

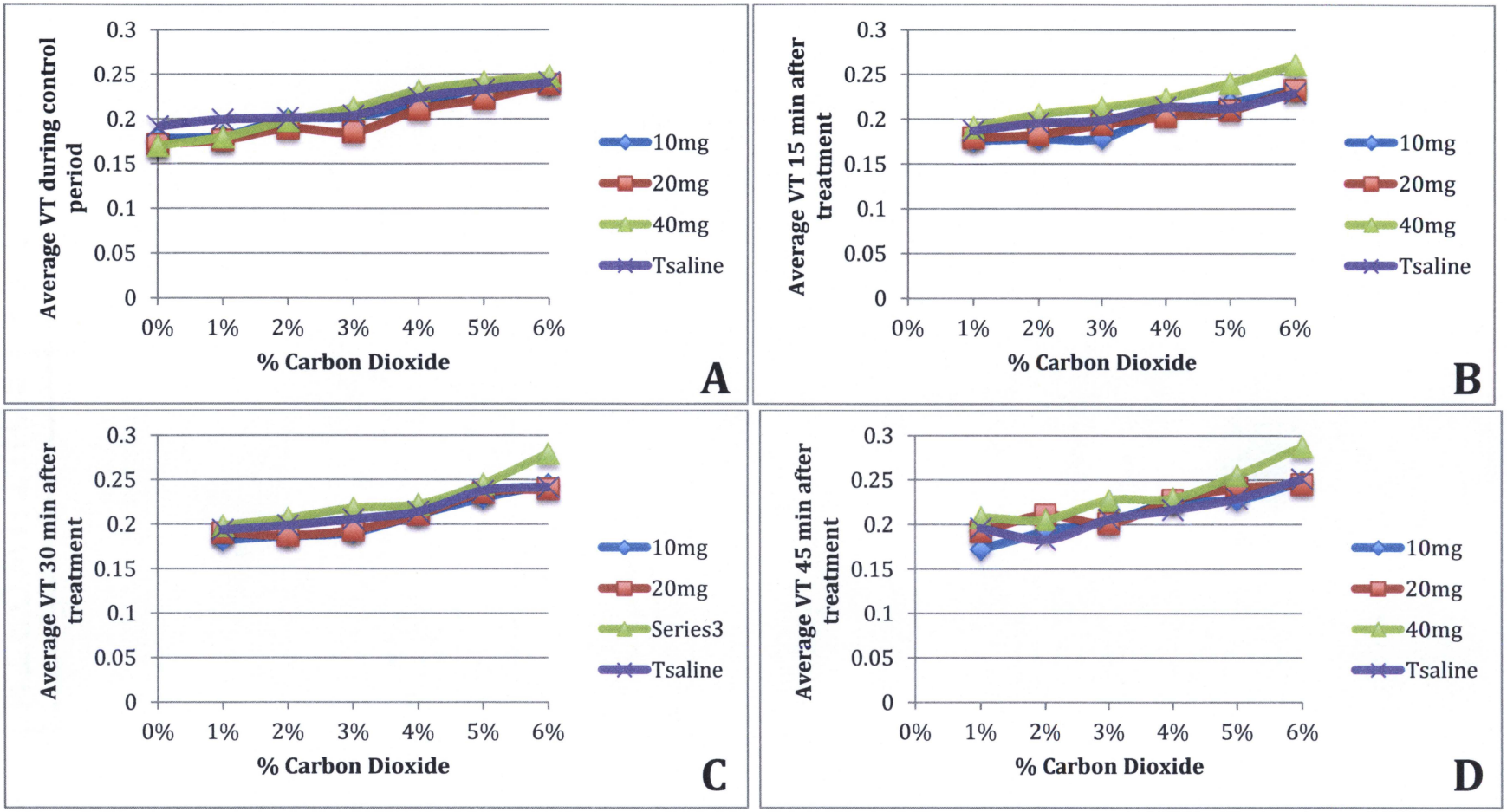

Fig. 18. The effect of THEO on the average tidal volume response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection

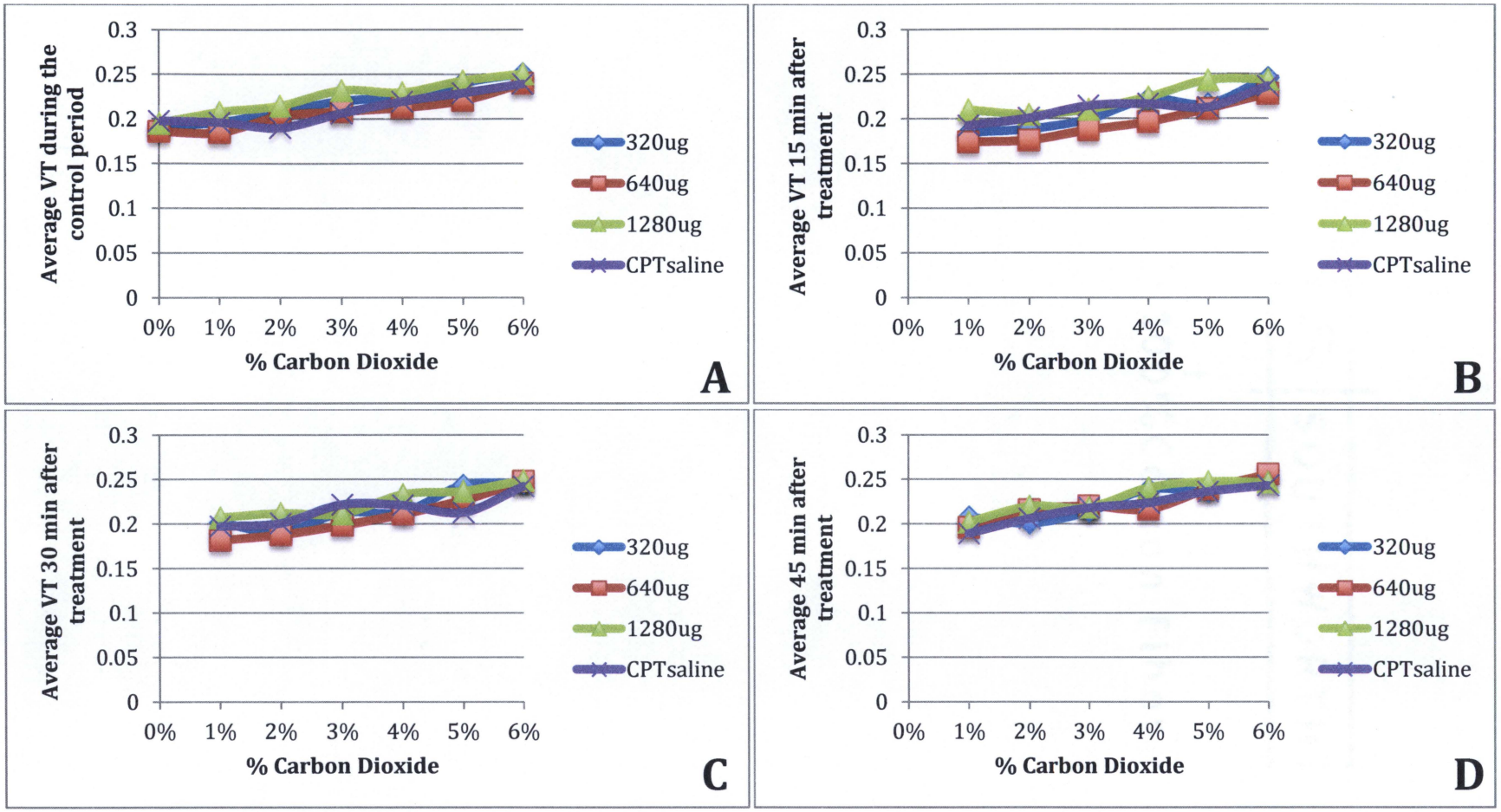

Fig. 19. The effect of CPT on the average tidal volume response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection

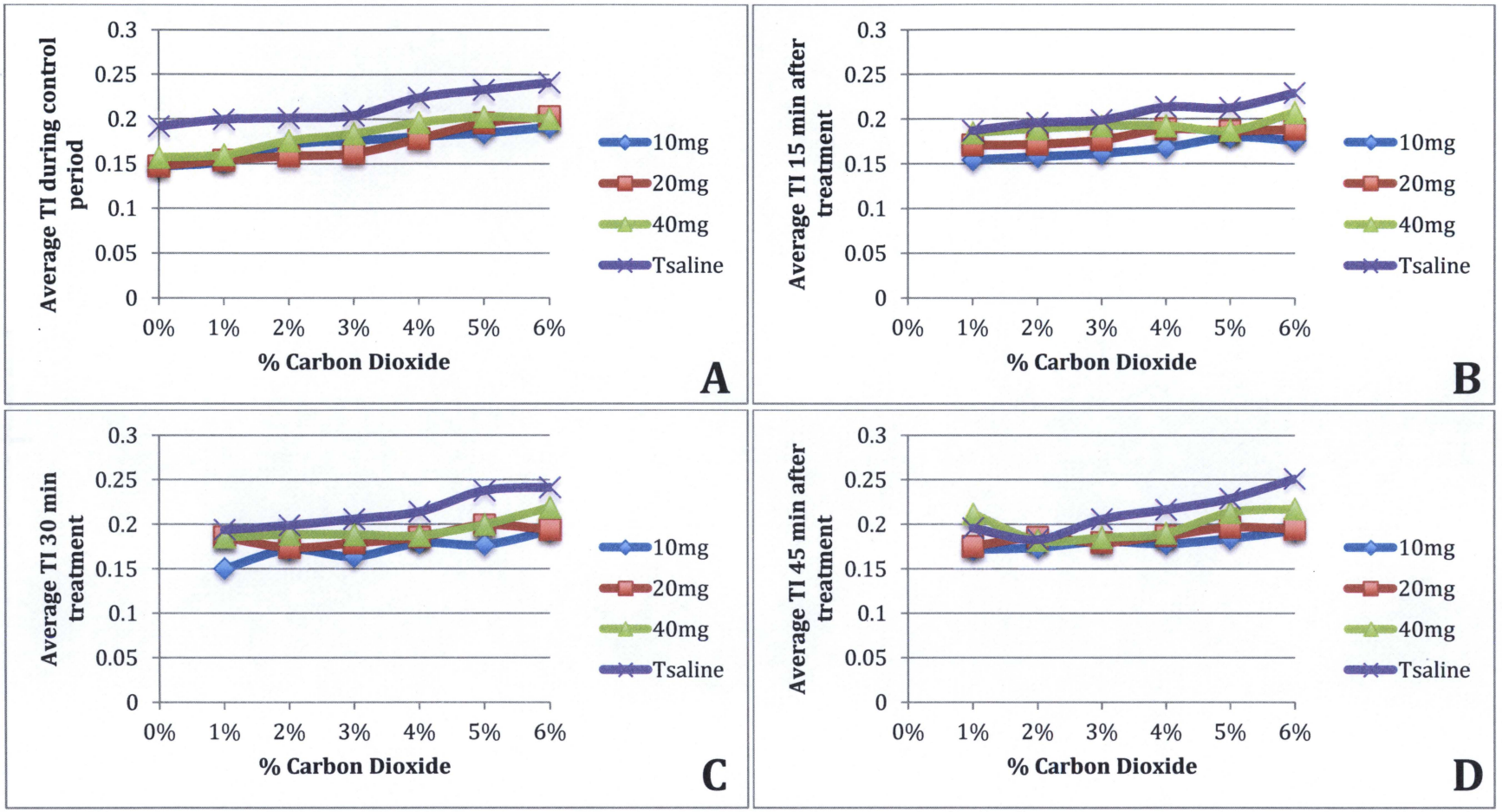

Fig. 20. The effect of THEO on the average time of inspiration response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection

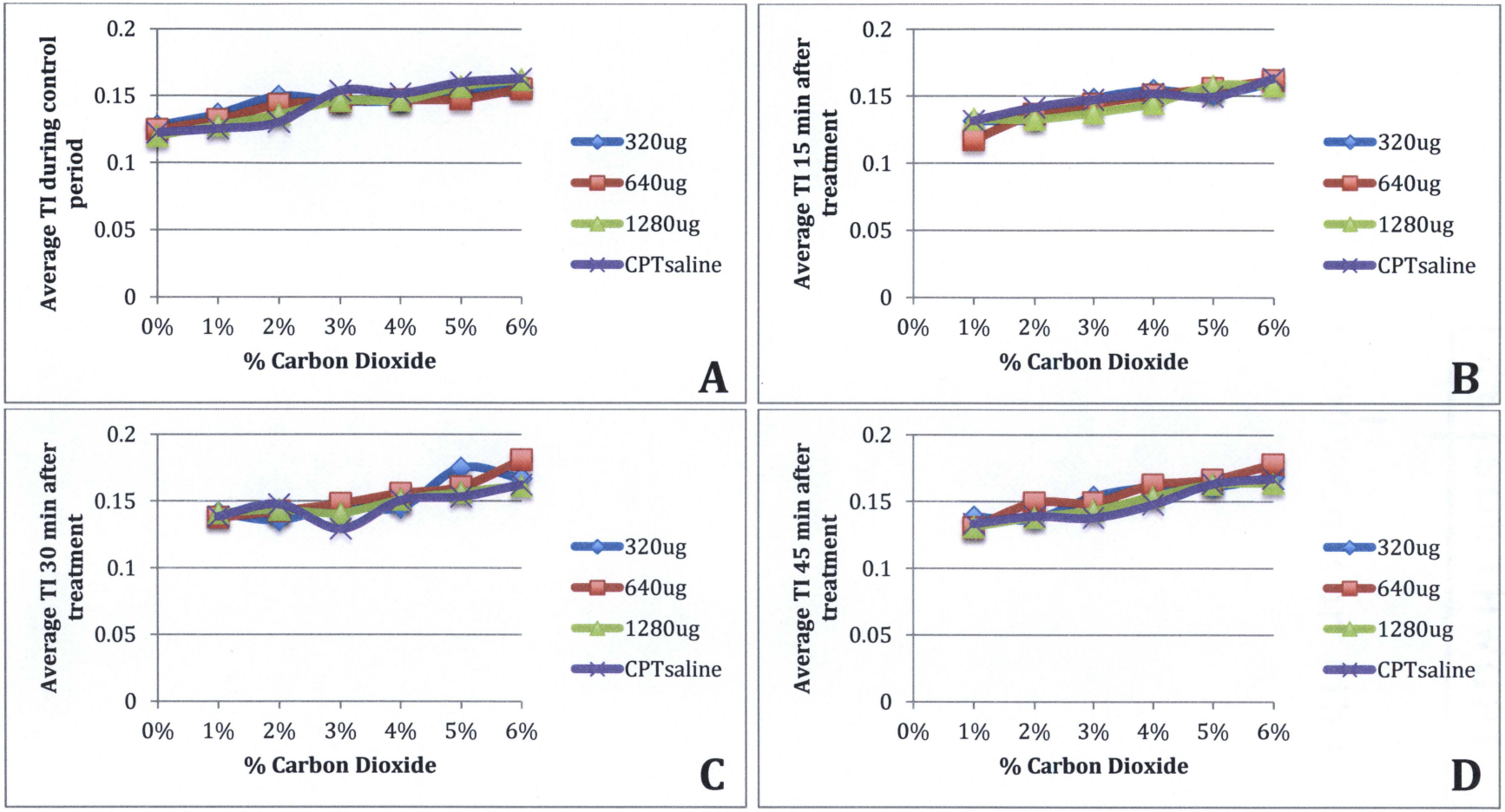

Fig. 21. The effect of CPT on the average time of inspiration response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection

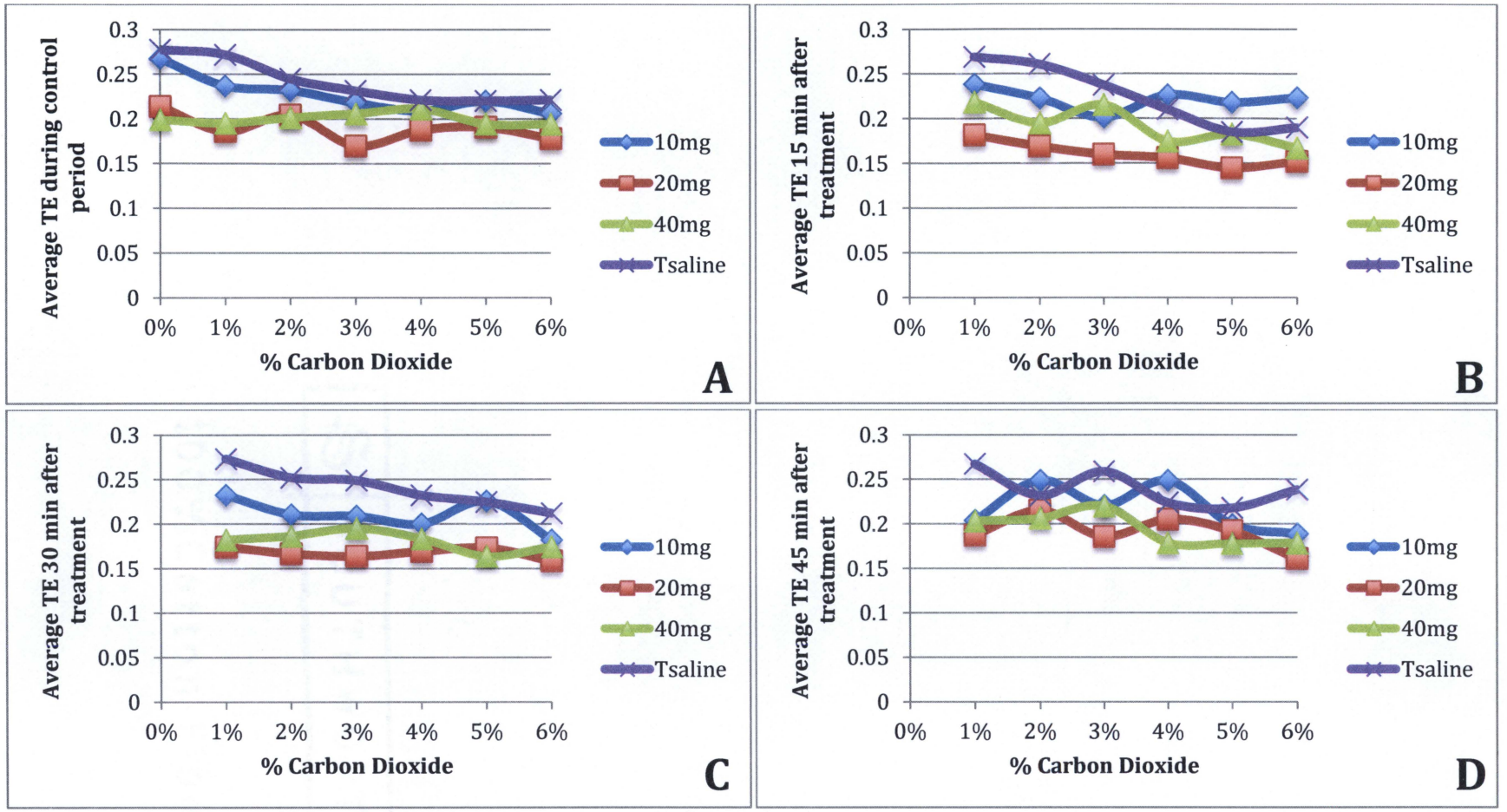

Fig. 22. The effect of THEO on the average time of expiration response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection

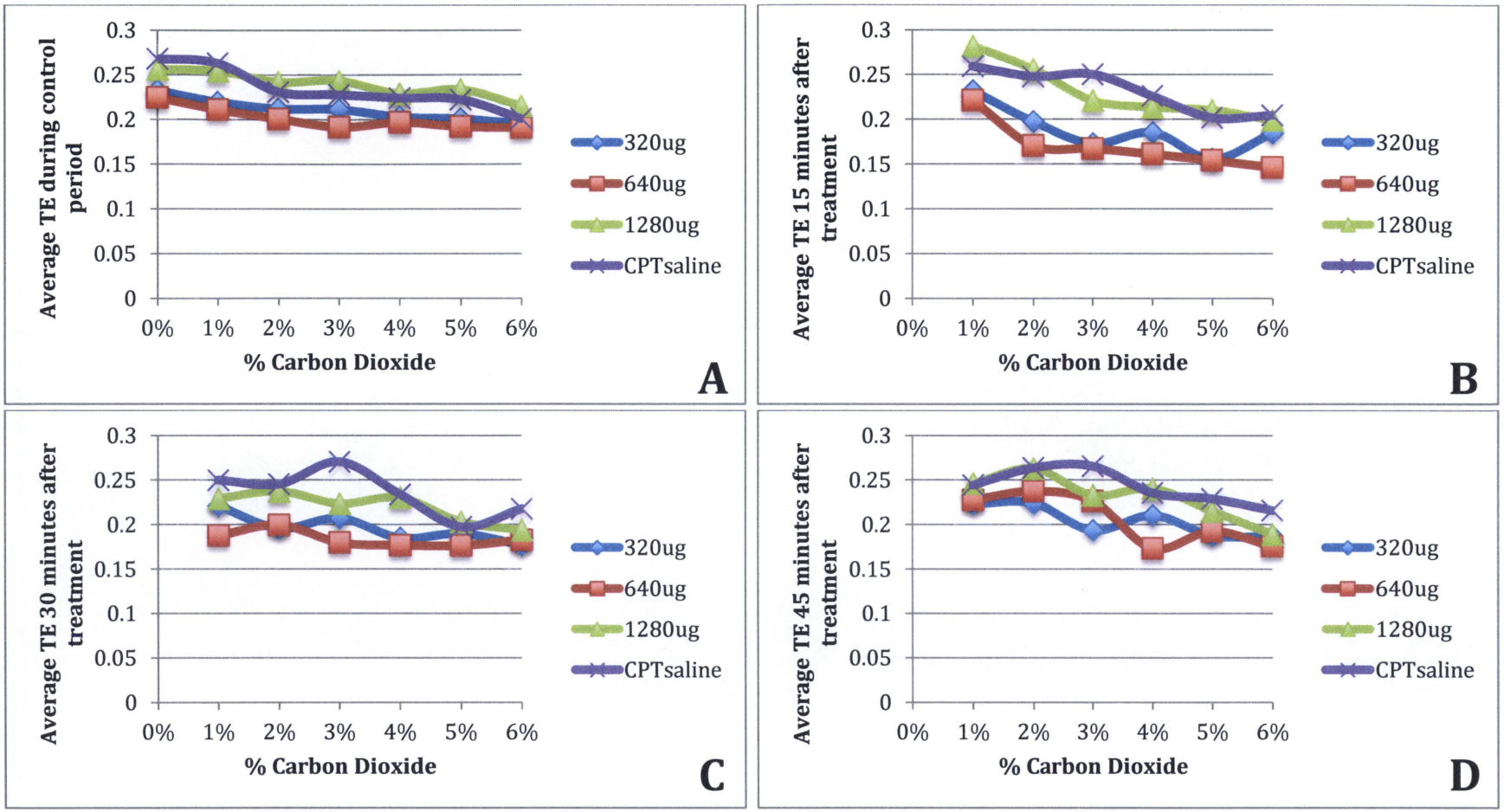

Fig. 23. The effect of CPT on the average time of expiration response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection

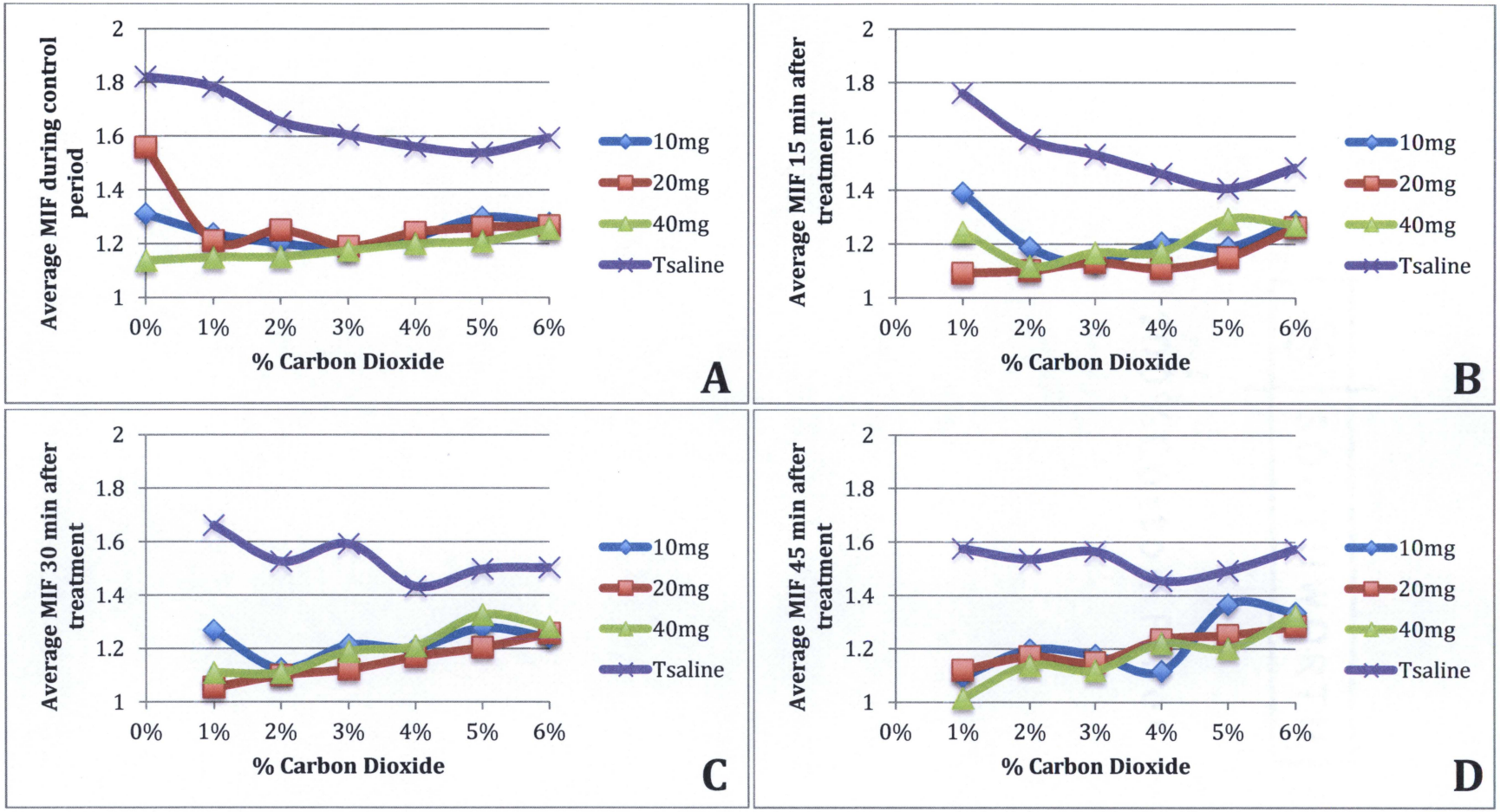

Fig. 24. The effect of THEO on the average mean inspiratory flow response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection

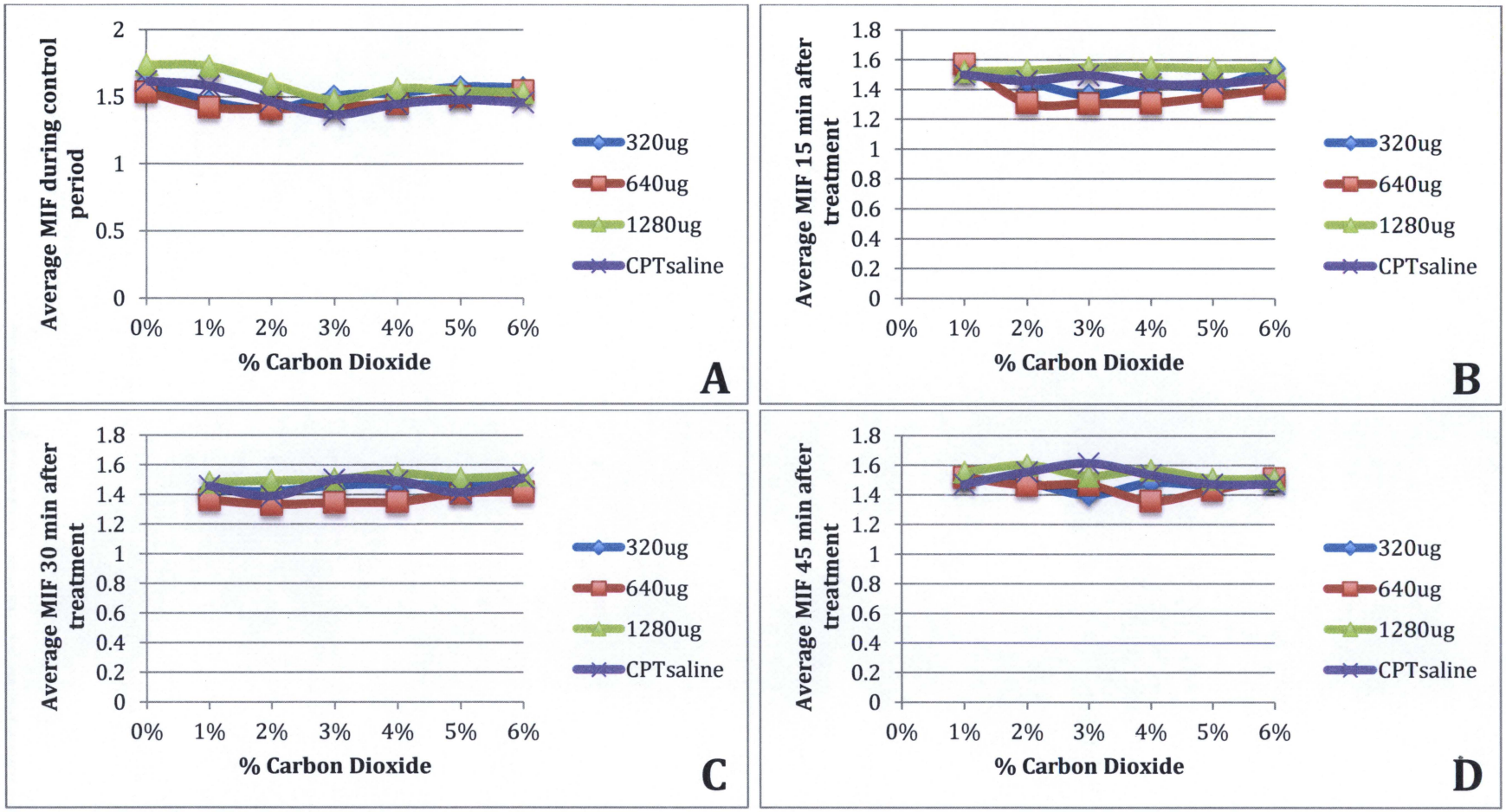

Fig. 25. The effect of CPT on the average mean inspiratory flow response to CO_2 at four different time periods, predrug (A), 15 min (B), 30 min (C), and 45 min (D) after drug injection.

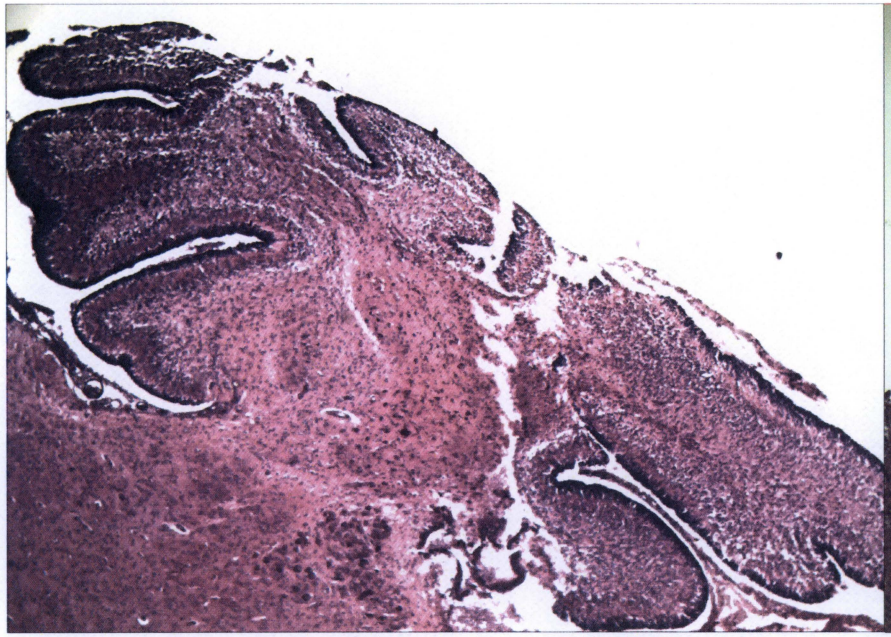

Fig. 26. Section of a four-day-old white rat (*Rattus norvegicus*) brainstem (scale set at 100 μm).

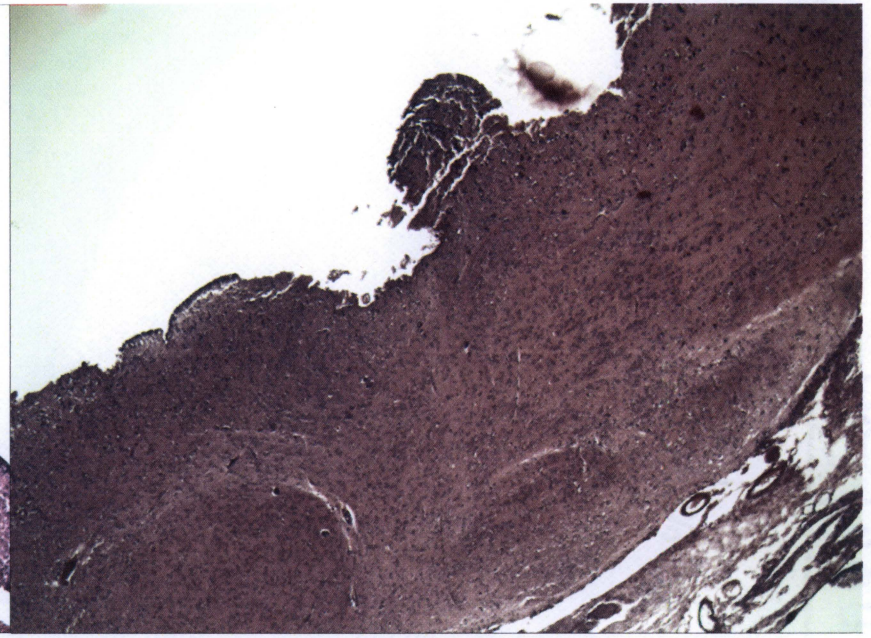

Fig. 27. Section of a 7-day-old white rat (*Rattus norvegicus*) brainstem (scale set at 100 μm).

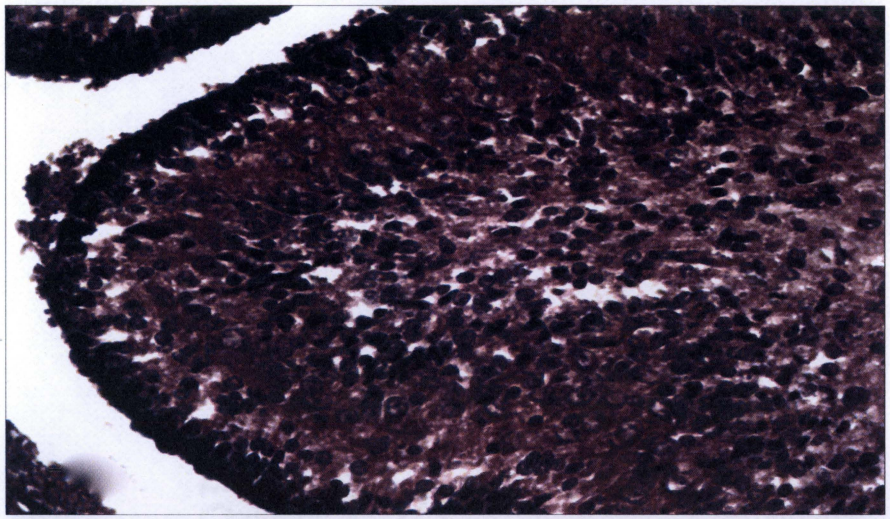

Fig. 28. Section of a 7-day-old white rat (*Rattus norvegicus*) brainstem (scale set at 50 μm).

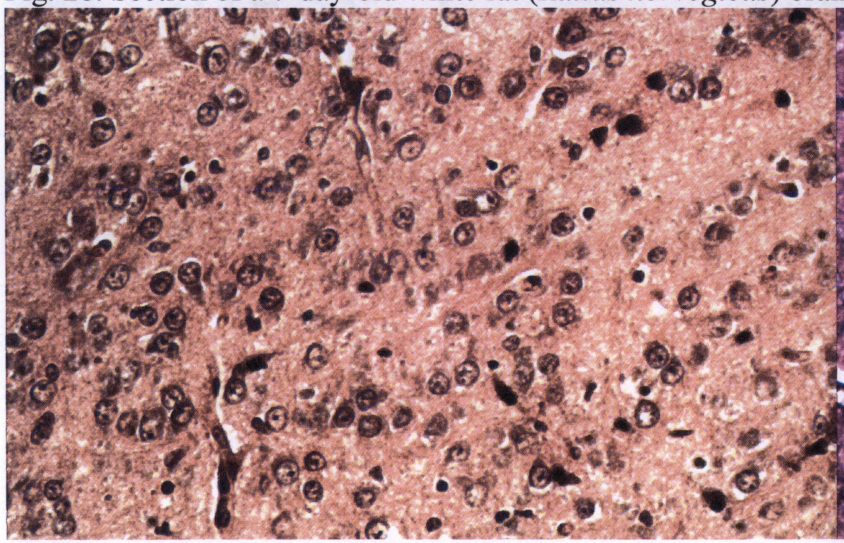

Fig. 29. Section of a 4-day-old rat (*Rattus norvegicus*) brainstem (scale set at 50 μm).

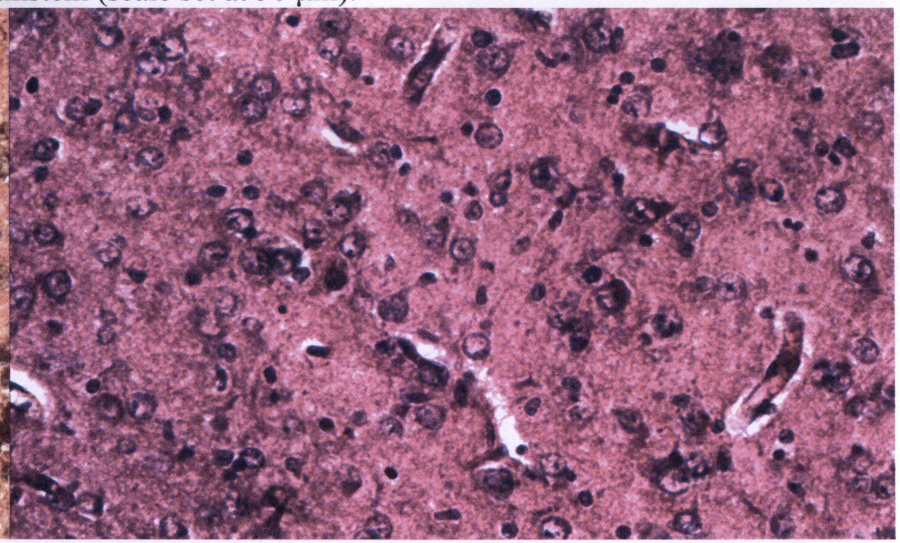

Fig. 30. Section of a 7-day-old rat (*Rattus norvegicus*) brainstem (scale set at 50 μm).